LINOLEIC ACID AND β-GLUCAN QUANTIFICATION OF TIGER MILK MUSHROOM AND ITS EFFECT ON BEAS-2B LUNGS EPITHELIAL CELL LINE

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by

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

%	Percentage
µg/ml	Microgram per milliliter
µm	Micrometer
ATP	Adenosine triphosphate
BEAS-2B	Human bronchial ephitelial lung cell line
CAM	Complementary and alternative medicine
CWE	Cold water extract
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
g	Gram
x g	Relative centrifugal force
HE	Hexane extract
HPLC	High performance liquid chromatography
HRE	Hexane residue extract
HWE	Hot water extract
I.D.	Internal diameter
IFN-γ	Interferon-gamma
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-17A	Interleukin-17A
IL-17E	Interleukin-17E

IL-1β	Interleukin-1β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IFN-δ	Interferon-delta
NO	Nitric oxide
kb	Kilo base pairs
kHz	Kilohertz
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
ml	Milliliter
mm	Millimeter
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/B	Nota bene – note well
nm	Nanometer
^{o}C	Degree celcius
PEE	Petroleum ether extract
PRE	Petroleum ether residue
SPSS	Statistical Programme for Social Sciences
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha

WTS	Water soluble tetrazolium
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide
β-glucan	Beta-glucan

PENENTUAN ASID LINOLEIK DAN β-GLUKAN EKSTRAK CENDAWAN SUSU HARIMAU DAN KESANNYA TERHADAP SEL SELANJAR EPITELIUM BEAS-2B

ABSTRAK

Lignosus rhinocerus merupakan polipor yang terdapat di hutan tropika Malaysia telah digunakan secara tradisi oleh penduduk tempatan untuk merawat pelbagai penyakit seperti batuk kronik, lelah, kanser payudara serta digunakan sebagai tonik kesihatan untuk menguatkan sistem imun. Tujuan kajian semasa ini dijalankan adalah untuk mengenalpasti fitokimia yang berpotensi dan mengkaji kesannya terhadap sel selanjar epitelium bronkus BEAS-2B. *L. rhinocerus* telah diekstrak menggunakan pengekstrakan soxhlet, pemaseratan dan pengekstrakan pelarut dengan bantuan ultrabunyi. Kehadiran asid linoleik di dalam ekstrak ditentukan menggunakan kromatografi cecair prestasi tinggi (HPLC) manakala kehadiran β-glukan pula ditentukan menggunakan 'Megazyme Yeast and Mushroom β-glucan Assay Kit'. Ekstrak ini kemudiannya telah melalui beberapa ujian seperti ujian sitotoksisiti menggunakan 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS). Penentuan apoptosis menggunakan sitometri aliran manakala analisis profil sitokin seperti interleukin-2, -4, -5, -6, -10, -12, -13, -17A, interferon gamma, faktor nekrosis tumor alfa, faktor transformasi pertumbuhan beta dan faktor ransangan koloni granulosit pula menggunakan kaedah asai imunojerapan berpaut enzim (ELISA). Dalam kajian ini, kaedah HPLC yang mudah telah dioptimumkan untuk pengesanan asid linoleik dalam ekstrak *L. rhinocerus*. Pemisahan dicapai menggunakan turus fasa terbalik C_{18} (250 × 4.6 mm, 5 μm) dan pengesanan dijalankan menggunakan pengesan ultraungu pada 208 nm. Fasa bergerak yang digunakan adalah gabungan asetonitril, metanol

dan heksana dalam nisbah 90:8:2 dengan penambahan 0.2% asid asetik pada kadar aliran 1.0 ml/min. Umumnya, ekstrak tidak polar seperti heksana dan petroleum eter menghasilkan jumlah asid linoleik yang lebih tinggi berbanding dengan ekstrak berasaskan air. Analisis komposisi β-glukan dalam ekstrak *L. rhinocerus* yang berasaskan air menunjukkan ekstrak residu heksana menghasilkan komposisi β-glukan tertinggi berbanding ekstrak lain. Dalam kajian ini, kebanyakan ekstrak (air panas, heksana, residu heksana dan residu petroleum eter) tidak mempamerkan kesan sitotoksik terhadap sel BEAS-2B kerana penurunan kadar kebolehhidupan sel adalah tidak melebihi 30%. Ekstrak air sejuk dan petroleum eter menunjukkan kesan sitotoksik ringan pada kepekatan 62.5-250.0 μg/ml. Analisa statistik menunjukkan bahawa rawatan dengan ekstrak-ekstrak *L. rhinocerus* tidak mengganggu aktiviti apoptosis sel BEAS-2B menandakan bahawa rawatan dengan ekstrak *L. rhinocerus* adalah tidak memudaratkan memandangkan disregulasi apoptosis dalam sel bukan sasaran boleh menyebabkan masalah kesihatan yang berat seperti kanser. Kesemua ekstrak ini juga tidak memberi kesan kepada rembesan sitokin yang diuji menandakan bahawa *L. rhinocerus* tidak memberi peranan yang penting dalam tindakbalas keradangan. Kesimpulannya, *L. rhinocerus* adalah ubat tradisional yang tidak memudaratkan. Penyiasatan lanjut perlu dilakukan terhadap cendawan ini memandangkan ianya menunjukkan potensi yang baik agar kesan terapeutiknya terhadap pelbagai penyakit dapat difahami dengan lebih mendalam.

LINOLEIC ACID AND β-GLUCAN QUANTIFICATION OF TIGER MILK MUSHROOM AND ITS EFFECT ON BEAS-2B LUNGS EPITHELIAL CELL LINE

ABSTRACT

Lignosus rhinocerus, a polypore found in the tropical forests of Malaysia is traditionally used by the locals to treat various diseases such as chronic cough, asthma, breast cancer as well as a health tonic to strengthen immune system. The aim of the current study is to identify potential phytochemicals and investigate their effects on BEAS-2B bronchial epithelial lung cell line. *L. rhinocerus* was extracted using soxhlet extraction, maceration and ultrasound assisted solvent extraction. Water was used as solvent in all methods. Hexane and petroleum ether were also used in ultrasound assisted solvent extraction. The extracts were then quantified for the presence of linoleic acid using high performance liquid chromatography (HPLC) and β-glucan using Megazyme Yeast and Mushroom β-glucan Assay Kit. These extracts were then subjected to cytotoxicity assay by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS). Apoptosis assay was determined using a flow cytometry while cytokine profile analysis of interleukin-2, -4, -5, -6, -10, -12, -13, -17A, interferon gamma, tumor necrosis factor alpha, transforming growth factor and granulocyte colony-stimulating factor was conducted by an enzyme-linked immunosorbent assay (ELISA). In this study, a simple HPLC method was optimized for detection of linoleic acid in *L. rhinocerus* extracts. Separation was achieved on a reversed-phase C_{18} column (250 × 4.6 mm, 5 μm) and the detection was done by an ultraviolet detector at 208 nm. The mobile phase used was a combination of acetonitrile, methanol and hexane in the ratio of 90:8:2 with the addition of 0.2% acetic acid at a flow rate of 1.0 ml/min. Generally,

non-polar extracts such as hexane and petroleum ether yielded a significantly higher amount of linoleic acid as compared to water-based extracts. Analysis of β-glucan in *L. rhinocerus* extracts indicated that the hexane residue yielded the highest β-glucan level compared to the other extracts. In this study, the majority of the extracts (hot water, hexane, hexane residue and petroleum ether residue) were not cytotoxic against BEAS-2B cell line since there was only < 30% reduction in cell viability. Cold water and petroleum ether extracts exhibited mild cytotoxic effect at the concentration of 62.5-250.0 μg/ml. Statistical analysis ($\alpha < 0.05$) showed that the treatment with *L. rhinocerus* extracts did not disturb the apoptosis activity of BEAS-2B cells indicating that it is not harmful since dysregulation apoptosis in non-targeted cell will lead to serious health problem such as cancer. These extracts also demonstrated no effect on the cytokines secretion suggesting that it does not play an important part in inflammatory response in BEAS-2B. In conclusion, *L. rhinocerus* is a harmless traditional remedy. Further investigation should be done on this mushroom which showed good potential in order to have a better understanding on its therapeutic potential on various diseases.

CHAPTER 1

INTRODUCTION

1.1 Inflammation and inflammatory lung diseases

Inflammation is the body's response towards insults including infection and injury (Medzhitov, 2008). It involves the interaction between various cell types and the production of many chemical mediators. Upon stimulation, the inflammatory cells at the affected areas secrete a variety of cytokines, chemokines, lipid mediators and bioactive amines (Coutinho and Chapman, 2011). During inflammation, leukocytes accumulate and amplify the response although excessive or prolonged inflammation can be damaging to the host. In normal circumstances, the immune system has several mechanisms to overcome the inflammatory responses. The resolution of inflammation requires the abortion of pro-inflammatory signalling pathways and clearance of inflammatory cells, allowing the restoration of normal tissue function where failure of these mechanisms leads to chronic inflammation and disease (Lawrence and Fong, 2010). Lung diseases including asthma, chronic obstructive pulmonary disease, and acute respiratory distress syndrome are the result of unresolved inflammation (Moldoveanu *et al.*, 2009).

In inflammation, there are two groups of cytokines namely pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines are involved in the activation of the immune system and participate in the inflammatory response. Examples of pro-inflammatory cytokines are interleukin 1 beta (IL-1β), IL-6, IL-8, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α). Meanwhile, anti-inflammatory cytokines including IL-10 and transforming growth factor beta (TGF-β) are involved in the down regulation of inflammatory response in the lungs

(Moldoveanu *et al.*, 2009). There are many evidences that associate cytokines with pulmonary diseases. For instance, IL-1β, IL-6, IFN-γ, granulocyte-macrophage colony-stimulating factor and TNF-α involve in chronic obstructive pulmonary disease (Sarir *et al.*, 2008). The levels of TNF-α and IL-1β are increased in people with acute respiratory distress syndrome (Suter *et al.*, 1992). In addition, the levels of TNF-α, IL-1β, and IL-6 in the sputum and bronchoalveolar lavage fluid of are reported to be increased (Barnes, 2008).

Currently, morbidity and mortality rates of lung diseases are alarming with four millions of people worldwide losing their lives annually and prematurely due to chronic respiratory diseases (Ferkol and Schraufnagel, 2014). It is estimated that 250 000 people die every year due to asthma and the number is increasing and is expected to reach 400 million by 2025 (Pawankar, 2014). Although asthmatic symptoms are more prevalent in high-income countries, the trend is also seen in some low- and middle-income countries (Beran *et al.*, 2015). In 2011, more than 2.7 million American adults experienced chronic obstructive pulmonary disease with about 135 000 deaths reported (Adeloye *et al.*, 2015). Since 1967, acute respiratory distress syndrome is accepted as the leading clinical respiratory problem worldwide. It is estimated that the prevalence of acute respiratory distress syndrome in high- and middle-income countries vary from 10.1 to 86.2 per 100,000 person-years (Riviello *et al.*, 2016). However, there is no clear evidence on the prevalence of acute respiratory distress syndrome in low-income countries due to under diagnosis (Riviello *et al.*, 2016).

The data on the prevalence of respiratory diseases from South-East Asia regions are still sparse. According to WHO (2014) in noncommunicable diseases country profiles, 7% of mortality among Malaysian population is attributed to

chronic respiratory diseases and the rates are decreasing from 2000-2012. According to Ministry of Health (2016) heath fact, respiratory diseases are among the ten principle causes of hospitalisation with 12.34% in general hospitals and 14.79% in private hospitals. Respiratory diseases are also among the principle causes of death in general and private hospital with 18.54% and 13.50% respectively.

1.2 Drugs and treatment for inflammatory lung diseases

To date, various anti-inflammatory drugs are used in the treatment of inflammation of lung diseases. These include corticosteroids, mast cell stabilizers and anti-immunoglobulin E antibody as well as leukotriene receptor antagonist. Corticosteroids are glucocorticoids that can inhibit many cells involved in inflammatory response in the lungs such as eosinophil, T-lymphocytes, mast cell and dendritic cells (Barnes and Adcock, 2003). Corticosteroids have clinically significant effects on asthma but are found to be less effective in chronic obstructive pulmonary disease (Donohue and Ohar, 2004). Although oral corticosteroids are the most effective treatment available, inhaled corticosteroids are better because it can minimize systemic adverse effects such as osteoporosis, cataract formation and muscle weakness (Walsh *et al.*, 2001). However, long term consumption of moderate and high dosage of inhaled corticosteroids will increase the risk of adverse effect (Tattersfield *et al.*, 2004).

Mast cell stabilizing drugs such as nedocromil and cromolyn inhibit the release of allergic mediators such as histamine, leukotrienes, and cytokines from the mast cell and are used to prevent allergic reaction (Finn and Walsh, 2013). Another mast cell stabilizing drug is omalizumab. Omalizumab is an accepted treatment for moderate to severe asthma (D'Amato *et al.*, 2007). Nedocromil, cromolyn and omalizumab are used to treat asthma but not chronic obstructive pulmonary disease

because problems with mast cells do not appear to be part of the clinical feature of the latter.

Another important anti-inflammatory medication used to treat lung disease is leukotriene receptor antagonist. This medication is used to block the effects of leukotrienes as part of the inflammatory cascade. Currently available leukotriene receptor antagonists are pranlukast hydrate, zafirlukast, zileuton and montelukast. Pranlukast hydrate, zafirlukast, and montelukast have bronchodilator effects (Tamada and Ichinose, 2017). However, many of these drugs produced severe side effects. For example, zileuton can cause liver injury (Harrill *et al.*, 2017) indicating the need of finding alternative therapies.

1.2.1 Complementary and alternative medicine for inflammatory lung diseases

To date, complementary and alternative medicine (CAM) is gaining attention among the public due to its effectiveness and minimal adverse effects. The examples of CAM that may complement modern medicine are including acupuncture, herbal medicine, aromatherapy, homeopathy, massage, traditional Chinese medicine and yoga. CAM are often utilized by people with chronic conditions where the available choices of conventional treatment are limited (McFarland *et al.*, 2002). Interestingly, although most of the patients are aware of the fact that CAM intervention is not necessarily curative, the preference towards the treatments is surprisingly high.

Many literatures have shown the effectiveness of CAM. For example, a study on the effects of the purple passion fruit peel extract on asthmatic patients showed a reduction of wheeze, cough and shortness of breath among patients who received oral administration of purple passion fruit peel (Watson *et al.*, 2008). Another research on asthmatic patients demonstrated that South African geranium can effectively treat acute respiratory infections (Timmer *et al.*, 2013) while reducing

asthmatic attack during upper respiratory tract viral infections in asthmatic children (Tahan and Yaman, 2013). In another study, extracts of *Siegesbeckia glabrescens*, reduced mucus overproduction in airways in an asthma murine model, decrease the expression of inducible nitric oxide (NO) and cyclooygenase-2, thus reducing the number of inflammatory cells in bronchoalveolar lavage fluid and the cytokine release (IL-4, IL-5 and IL-13) (Jeon *et al.*, 2014). Lavender essential oil can reduce airway resistance and the eosinophils count recovered in brochoalveolar lavage fluid and also in peribronchial and perivascular tissues in a murine model of bronchial asthma. Furthermore, the oil reduced mucous cell hyperplasia and the messenger ribonucleic acid expression of mucin 5b without significantly changing the messenger ribonucleic acid expression of mucin 5ac. Mucin 5b and mucin 5ac are two of the most secreted mucin in airways (Ueno-Iio *et al.*, 2014).

1.3 Natural products

Natural products are wide range of chemical entities produced in nature by living organisms such as plant, animal and microorganism (Katz and Baltz, 2016). They have been important part in health care and prevention of disease for thousands of years. This is supported by written evidence from the ancient civilizations of China, India and North Africa (Phillipson, 2001). In addition, a 4000 year old Sumerian clay tablet also recorded many remedies for various illnesses (Kong *et al.*, 2003). To date, natural products are still being used as alternative medicines in all over the world.

1.4 Mushrooms

Since the isolation of quinoid pigments from mushrooms fruiting body in 1877, scientists have embraced mushrooms as an important source of bioactive compounds (Stadler and Hoffmeister, 2015). Apart from having abundant carbohydrates, proteins, free amino acids, vitamins, minerals and trace elements (Colak *et al.*, 2009; Kalač, 2013) mushrooms are also rich in bioactive compounds including polysaccharides, ergosterols, phenolics, polyphenolics, terpenoids, lectins, and volatile organic compounds (He *et al.*, 2012; Villares *et al.*, 2012; Wang and Marcone, 2011). Mushroom extracts have several medicinal properties such as anticancer (Zong *et al.*, 2012), antibacterial (Zong *et al.*, 2012) and antiviral (Adotey *et al.*, 2011) properties. In addition, they also have antioxidant (Mu *et al.*, 2012), antihypoglycaemic (Lo and Wasser, 2011) as well as anti-atherosclerotic agents (Guillamón *et al.*, 2010). They also have immunomodulatory properties (El Enshasy and Hatti-Kaul, 2013). Moreover, the presence of many compounds with anti-inflammatory effects has been reported. In addition, many compounds of highly diversified chemical structures with anti-inflammatory activities against inflammatory mediators such as NO, cytokines, and prostaglandins have been isolated and purified from different types of mushrooms (Elsayed *et al.*, 2014) indicating their potential to be used against inflammation.

1.5 *Lignosus rhinocerus*

Lignosus rhinocerus is a polypore found in the tropical forests of Malaysia. It is known as “cendawan susu rimau” or “tiger milk mushroom” among the locals. The mushroom gained its unique name from folklore where it is believed that it grows from the ground where a tigress spills her milk during lactation. The local name is

also associated with the physical appearance of which is akin to a thickened mass of tiger's milk. *L. rhinocerus* is made up of pileus, stipe and sclerotium (Figure 1.1).

L. rhinocerus is utilised in Malay traditional medicine. It is usually consumed together with other Malay traditional medicines. In the earlier days, *L. rhinocerus* was utilized by the Malay community to cool the body, as a postpartum medicine for women following childbirth, as a contraceptive mushroom as well as to treat breast and cervical cancers (Lau *et al.*, 2015). It is reported that *L. rhinocerus* are still widely used nowadays by the indigenous community in Malaysia to cure asthma, cough, joint pain, food poisoning, swollen breast, liver-related illnesses cancer and as a general tonic (Lee *et al.*, 2009).

During a speech at the Convention on Biotechnology in 2002, Tun Dr Mahathir Mohamad was reported to have claimed that his chronic cough stopped following consumption of traditional remedy derived from *L. rhinocerus* (Wong L. Z., 2011). In addition, *L. rhinocerus* can boost energy, alertness and overall wellness of the user (Sabaratnam *et al.*, 2013). Interestingly, even the urban community in Malaysia utilized *L. rhinocerus* for its medical purposes (Lee and Chang, 2007). *L. rhinocerus* and its medicinal properties are also widely recognised by other communities outside Malaysia especially in other Asian regions such as Singapore, Thailand, Hong Kong, and China (Lau *et al.*, 2015).

There have been many efforts to confirm the medicinal properties of *L. rhinocerus* scientifically. Cold water extracts from the sclerotium of *L. rhinocerus* is reported to have anti-proliferative activity on human breast carcinoma (MCF-7) and human lung carcinoma (A549) (Lee *et al.*, 2012). The *in vivo* anti-inflammatory activity of *L. rhinocerus* sclerotium extracts has also been reported (Lee *et al.*, 2014).

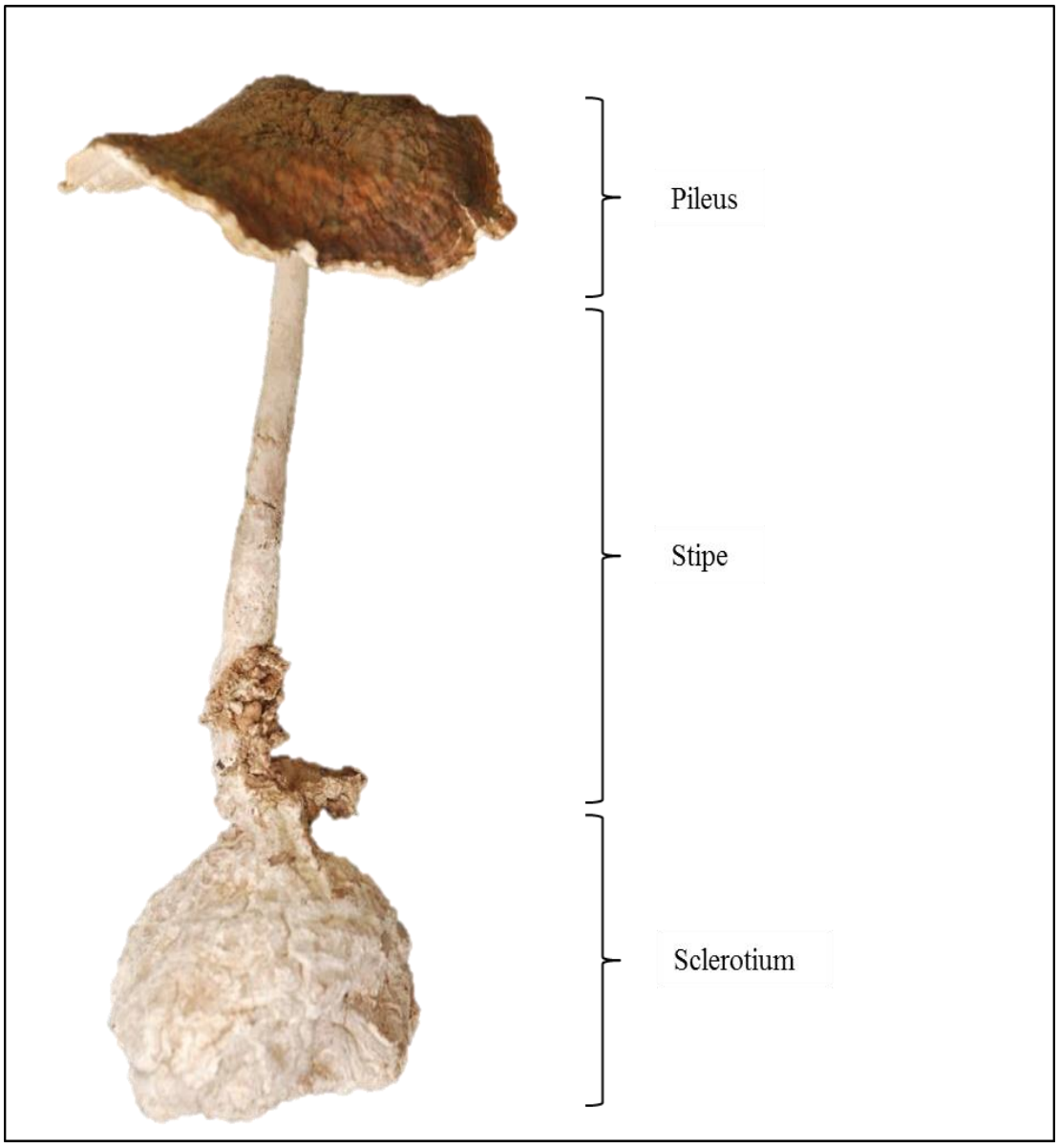

Figure 1.1: Structure of *L. rhinocerus*. (Adapted from: *https://phys.org/news/2013-12-precious-tigers-mushroom.html*)

In addition, *L. rhinocerus* is reported to exhibit *in vivo* anti-asthmatic effects (Johnathan *et al.*, 2016). The aqueous extract of *L. rhinocerus* sclerotium can enhance neurite outgrowth activity (Eik *et al.*, 2011). Lee *et al.* (2011) reported that there is no treatment-related sub-acute toxicity in rats after 28-days oral administration of different concentration of wild type *L. rhinocerus* sclerotial powder. A similar study by another group of scientists also showed no significant adverse effects following the administration of *L. rhinocerus* (Kong *et al.*, 2016) indicating its potential to be investigated as an anti-asthmatic agent.

1.5.1 Phytochemical analysis of *L. rhinocerus*

The medicinal properties of natural products are associated with their secondary metabolites such as alkaloids, terpenoids, phenolic, tannins, steroids and volatiles. These secondary metabolites make natural products remarkable sources of potential therapeutic agents against various diseases. Some of the metabolites can also act as sedative, analgesic, cardiotonic agents, anti-inflammatory, oxytoxics and as immune modulators (Okwu and Iroabuchi, 2009). In general, mushrooms are reported to have high mineral content, low fat and low calories (Lee *et al.*, 2011).

In case of *L. rhinocerus*, there is still limited data available which may be attributed to the fact that *L. rhinocerus* is very hard to be found in the wild. It is only recently that scientists develop cultivation method for *L. rhinocerus*. Hence, extensive studies on this subject are very much in need with the sustainability of its source. Currently, available data focus on the chemical compositions of the sclerotium since only this part is claimed to have medicinal properties. Lau *et al.* (2013) has conducted an extensive analysis including the comparison of chemical composition of different parts of *L. rhinocerus* such as pileus, stipe and sclerotium. According to the study, the pileus, stipe and sclerotium of *L. rhinocerus* are rich in

carbohydrates but low in proteins and fat contents. The pileus and stipe of *L. rhinocerus* contain higher protein and fibre as compared to the protein and fibre content in its sclerotium. *L. rhinocerus* is also rich in β-glucans. Besides, *L. rhinocerus* contains moderate amount of energy with low vitamin levels. Only riboflavin and niacin are found in levels are above detection limit. In addition, *L. rhinocerus* contains varying levels of all essential amino acids (Lau *et al.,* 2013) suggesting its nutraceutical potential which is not limited to the sclerotium only.

A gas chromatography-mass spectrometry analysis on the volatile constituents of *L. rhinocerus* showed that alkanes constitutes 53% of volatile components of *L. rhinocerus* followed by fatty acids (36%), benzene (7%), phenol (3%) and dicarboxylic acid (1%). Linoleic acid constitutes the major propotion in *L. rhinocerus*'s sclerotial hot water extract which is at 21.35 %, followed by 2,3-dihydroxypropyl elaidate (11.82%) and octadecane (10.47%) (Johnathan *et al.*, 2016).

1.5.1(a) Linoleic acid

Linoleic acid is the most abundant polyunsaturated fatty acid in *L. rhinocerus* and represents about 21.35% of total volatile compounds (Johnathan *et al.*, 2016). Linoleic acid is an omega-6 polyunsaturated fatty acid. It is the precursor for arachidonic acid, the other omega-6 fatty acid that is often associated with inflammation. Although many studies on linoleic acid and its derivatives indicate association with inflammation (Choque *et al.,* 2014), the findings remain inconclusive. For example, in a study examining the relationship of plasma polyunsaturated fatty acid with circulating inflammatory markers in humans, individuals with the lowest total omega-6 polyunsaturated fatty acid in the plasma had been reported to have the highest levels of pro-inflammatory cytokines such as

TNF-α and IL-6 and the lowest levels of anti-inflammatory cytokines including IL-10 (Ferrucci *et al.*, 2006). However another study examining the relationship between the intake of linoleic acid and alpha-linolenic acid with various inflammatory markers, consumption of omega-6 fatty acids is suggested not to inhibit the anti-inflammatory effects of omega-3 fatty acids and therefore the intake of both omega-6 and omega-3 polyunsaturated fatty acids were associated with the lowest levels of inflammation (Pischon *et al.*, 2003). A randomized cross-over design study to determine the effect of reducing linoleic acid intake on circulating IL-6 and C-reactive protein nevertheless failed to show any significant changes in both inflammatory markers (Liou *et al.*, 2007).

There are also a few studies on the potential impact of arachidonic acid on inflammation. A study on the increase in daily arachidonic acid intake to the diet of healthy individuals had no noticeable effect on the production of TNF-α, IL-1β, or IL-6 (Kelley *et al.*, 1998). Other similar study also reported that the addition of 700 mg of arachidonic acid per day to the habitual diet for 12 weeks failed to significantly increase the *ex vivo* production of similar pro-inflammatory cytokines (Thies *et al.*, 2001).

1.5.1(b) β-Glucan

Studies on the chemical compositions of *L. rhinocerus* indicated that it is rich in β-glucan which is known to have anti-inflammatory properties. Various evidence on the anti-inflammatory effects of β-glucans on inflammatory bowel disease exist (Ye *et al.*, 2011). In addition, β-glucan is also reported to have antioxidant and anti-tumour effects (Pelizon *et al.*, 2005). Many studies have reported the ability of β-glucans to activate leukocytes while stimulating cytotoxic, phagocytic and

antimicrobial activities (Du *et al.*, 2015). β-glucans vary in molecular weight, degree of branching and conformational structure all of which are believed to influence their biological properties. For instance, the fine structure, molecular weight, conformation and solubility of fungal β-glucans have been reported to influence many biological activities (Soltanian *et al.*, 2009). Another example is shown by the comparison study of different conformation of β-glucans on the synthesis of nitric oxide. From this study, it was reported that single helical β-glucans can enhance the synthesis of NO but not the triple helical β-glucans (Ohno *et al.*, 1996). In addition, it was found that insoluble glucan strongly stimulate inflammatory cytokine production (Ishibashi *et al.*, 2001).

Although there are many studies on the effect of β-glucan on respiratory health, the data available is disparate. One of the studies which reported that exposure to mould can lead to the development of asthma (Douwes, 2005) which contradicts to another data suggesting that β-glucan may play a protective role against asthma (Heederik and von Mutius, 2012). β-glucan has also been shown to have positive effect on upper respiratory tract infections (Fuller *et al.*, 2012). In addition to that, β-glucan significantly reduced pulmonary eosinophil influx and the production of T-helper 2 cytokines (IL-4, IL-5, IL-13) suggesting that it may be useful in targeting some aspects of asthma (Burg *et al.*, 2016).

1.6 Extraction and identification of natural product

Extraction of selected components from natural products for further separation and characterization is important. It included washing of the raw materials, drying and grinding. The pre-extraction processes are important to preserve the phytochemicals in the samples (Sasidharan *et al.*, 2011). Generally, both

fresh and dried samples can be used in the extraction. However, dried sample is preferable as fresh samples can get easily deteriorated (Sulaiman *et al.*, 2011).

Various extraction methods can be utilised including maceration, soxhlet extraction and ultrasound-assisted extraction. These methods were chosen because they are cheap and are easily available. Maceration is a method of soaking plant materials in a sealed container with a solvent followed by frequent agitation intended to soften and break the cell wall to release soluble phytochemicals. The choice of solvents will determine the type of compound extracted from the samples. Maceration technique is suitable for thermolabile compounds (Ncube *et al.*, 2008). In soxhlet extraction, finely ground sample is placed in a porous container called thimble. The solvent is placed in the bottom flask. When the bottom flask is heated, the solvent will vapourise and condense thus dripping into the extraction chamber; soaking the thimble. The liquid in the extraction chamber will be emptied into the bottom flask again when the liquid content reaches the siphon arm and the cycle continues. Ultrasound-assisted extraction involves the use of ultrasound ranging from 20 to 2000 kHz. The effect of ultrasound increases the permeability of cell walls of the sample, thus facilitating the release of compounds (Ncube *et al.*, 2008). Nevertheless, although the procedure is simple and cheap, not many laboratories have the facilities.

Besides the extraction method, the choice of solvents used also plays a vital part in the successful extraction of biologically active compounds. There are a few qualities that a good solvent should possess such as 1) low toxicity, 2) low boiling point, 3) promote quick absorption of the extract, 4) has preservative activity and 5) unable to cause the extract to complex or dissociate (Tiwari *et al.*, 2011). The choice of solvents is dependent on the type of desired compound to be extracted. There are

various selections of solvents that are commonly utilised in the extraction procedures. For instance, a universal solvent like water is commonly used to extract compounds with antimicrobial activity. However, the products of extraction using organic solvents are reported to have a better consistency in terms of antimicrobial activity (Das *et al.*, 2010). Polar solvents including methanol and ethanol are better at extracting hydrophilic components while non-polar solvent are good at extracting lipophilic compounds (Sasidharan *et al.*, 2011).

The presence of various bioactive compounds having different properties complicates the identification and characterization process. Therefore, a sensitive and specific detection method is important for proper identification of bioactive compounds. Currently, there are two identification techniques namely chromatographic and non-chromatographic techniques. An example of the chromatographic techniques is High Performance Liquid Chromatography (HPLC) while the non-chromatographic detection can be performed using Fourier Transform Infra Red.

1.6.1 High Performance Liquid Chromatography (HPLC)

HPLC is a versatile and robust method that has been widely used for separation and detection of natural products (Sasidharan *et al.*, 2011). There are several types of HPLC, each varying in the nature of the stationary phase. The first type is normal phase HPLC where the stationary phase is polar while the mobile phase is non-polar. Therefore, polar samples take longer time to be eluted as compared to the less polar samples (Younes *et al.*, 2016). The second type of HPLC is reverse phase HPLC. In-contrast to the normal phase, reverse phase has a nonpolar stationary phase and polar mobile phase hence the less polar material is generally retained longer (Meyer, 2013).

Another type of HPLC is called size-exclusion HPLC. In this system, the column is packed with materials that have precisely controlled pore size. The samples are separated according to the molecular size. Larger molecules are rapidly eluted from the column while smaller molecules are eluted later since they would penetrate inside the porous of the packing particles (Meyer, 2013). The fourth type of HPLC is ion-exchange HPLC. The stationary phase of this system has ionically charged surfaced with the charge opposite to the sample ions. The technique is suitable for ionic and ionisable samples and the retention time is dependent on the charge strength. Since samples with stronger charge will be strongly attracted to the ionic surface, it will take longer to elute (Meyer, 2013). In this study a reverse phase HPLC was chosen because it is rapid and flexible; applicable for separating a wide range of analytes. Moreover, the column is also highly stable and efficient.

There are few literatures reported on the use of HPLC in the detection of fatty acids. For example, HPLC was used in a study on the determination of free fatty acid in edible oils. In this study, the separation of fatty acid derivatives was carried out on a reversed-phase C_8 column (150 × 4.6 mm, 5 μm). The detection of fatty acid was monitored using online mass spectra equipped with an atmospheric chemical ionization source (Li *et al.*, 2011). HPLC was also used in the detection of free fatty acid from *Ganoderma lucidum*. Separation was achieved using C_{18} reversed phase column (25 cm × 4.5 mm, 5 μm) and the detection was monitored using evaporative light scattering detector (Hou *et al.*, 2017).

1.7 Bioassays

Bioassays are crucial to determine the biological activities of a substance. Bioassays involved the use of tissue or cell (*in vitro*) and animal (*in vivo*). The assay

is chosen based on the nature of the desired activity. An ideal bioassay would be sensitive, selective, cost effective and easy to run and maintain.

1.7.1 Cell proliferation assay

Cell proliferation is a process by which the cells grow and divide to replace lost cells. It is highly regulated in normal cells. On the other hand, some cell types, such as skin fibroblasts, smooth muscle cells, the endothelial cells that line blood vessels, continue to proliferate throughout life while other types including cardiac muscle cells are not able to undergo cell division. Most normal cells remain in a non-proliferative state unless they are stimulated to divide to replace the lost cells (Cooper, 2000). The pattern observed during cell proliferation recounts various interesting biological conditions that are useful for therapeutic and diagnostic purposes. Cell proliferation assay is also important in drug discovery as it tests drug toxicity (Yadav *et al.*, 2001). Thus, this assay is important to determine the cytotoxic effect of *L. rhinocerus* extracts on bronchial epithelial lung cell line (BEAS-2B) in order to ensure the safety of the *L. rhinocerus* extracts.

There are four types of cell proliferation assays with main difference in the parameters used to determine the proliferation rate. The parameters are such as the adenosine triphosphate (ATP) concentration, rate of deoxyribonucleic acid (DNA) synthesis, cell proliferation-related antigen and metabolic activity of cells (Romar *et al*., 2016). Proliferation rate can be observed by measuring the rate of DNA synthesis because DNA replication takes place at the start of cell division. Hence, the rate of DNA synthesis is directly proportional to the rate of cell proliferation. The rate of DNA synthesis can be analysed using ^{3}H-thymidine and 5-bromo-2′-deoxiuridine. ^{3}H-thymidine-based assays are highly sensitive. However, they are also radioactive

and are dangerous to the operator. Thus, 5-bromo-2′-deoxiuridine -based assay is regularly used (Romar *et al.*, 2016).

Another parameter to determine proliferation rate is assessment of certain antigens that are only present in proliferating cells by using antigen-specific antibodies. For example, the anti-Ki-6, is a popular antibody used in the measurement of protein expressed during the cell cycle in cancer research (Yadav *et al.*, 2001). Other markers that are frequently used in cell proliferation assays including proliferating cell nuclear antigen, topoisomerase IIB and phosphohistone H3 (Yadav *et al.*, 2001). The third parameter used to determine proliferation rate is by measuring the concentration of ATP in cells. Viable cells have the ability to synthesize ATP. As the cells lose their viability, membrane integrity is compromised which ceases the ability of cells to produce ATP. In another word, proliferation rate is directly proportional to the concentration of ATP in the cells and is measurable by using bioluminescence-based assay (Riss *et al.*, 2003).

Last but not least, cell proliferation activity can be measured by investigating the reduction potential of cells using either tetrazolium salts or resazurin dyes. During proliferation, the concentration of metabolic intermediates such as nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide, flavin mononucleotide and nicotinamide adenine dinucleotide increase (Riss *et al.*, 2016). These intermediates reduce tetrazolium salts to a formazan product resulting in colourimetric change. Likewise, resazurin will be reduced to resorufin (Riss *et al.*, 2016). The absorption of the media containing the dye solution can be read using a spectrophotometer or microplate reader. Common tetrazolium salts include 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-

tetrazolium (MTS) and 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (Riss *et al.*, 2003). Currently, proliferation assay that measure the concentration of ATP is the easiest, most sensitive, rapid and has the least amount of interference, however tetrazolium reduction assays offer cost effective substitutes that can achieve good performance depending on the experimental design (Riss *et al.*, 2016). Hence, MTS assay was selected to be used in this study.

1.7.2 Apoptosis assay

Apoptosis is a genetically regulated programmed cell death that involved three stages namely initiation, genetic regulation, and effector mechanisms (Renehan *et al.*, 2001). Various factors can initiate apoptosis such as the use of anticancer drugs, gamma and ultraviolet irradiations and survival factors (e.g: IL-1, TNF and Fas) deficiency (Andrew and Renehan, 2001). Abnormal rate of apoptosis leads to various biological conditions such as neurodegenerative and autoimmune diseases as well as cancer. The capability of substance to regulate the life or death of a cell is important, influencing its vast therapeutic potential (Elmore, 2007). Since apoptosis occur through a complex signalling cascade, there are many options of assays that can be used to analyse the activity of the proteins involved. However, since the results may overlap with that of necrosis, it is important to use more than one assay to ensure the accurate evaluation.

Early stage apoptosis is represented by the loss of mitochondrial membrane potential. Mitochrondrial membrane potential can be detected by JC-1, a dye that selectively enters the mitochondria. In functional mitochondria, JC-1 forms aggregates which show a fluorescent emission at 595 nm (Perelman *et al*., 2012). With reduced membrane potential the dye forms monomers where the emission is

shifted to 530nm. This shift is used to measure mitochondrial membrane potential, and the entry of the cell into early stage apoptosis (Perelman *et al*., 2012).

Another key apoptotic process is the translocation of phosphatidylserine, a protein located in the cellular membrane. Phosphatidylserine in live, intact cells is cytosolic but translocates to the extracellular portion of the membrane in the early apoptosis (Orrenius *et al*., 2010). Annexin V can be used for phosphatidylserine detection where cell impermeable dyes, such as Propidium Iodide, need to be used as a counterstain to differentiate early apoptosis from late apoptosis or necrosis (Rieger *et al*., (2011).

Late stage apoptosis can be identified by looking at the defragmentation of DNA. In apoptosis, DNA fragmentation occurs in two stages (Bicknell *et al.*, 1994) where the DNA is sequentially degraded into high molecular weight DNA fragments with an approximate size of 300 kb which can be detected by a gel electrophoresis (Higuchi, 2004). DNA feature of apoptotic DNA fragmentation is characterized by the production of both single and double stranded breaks. High molecular weight DNA fragments are considered as a reputable biochemical marker for apoptosis (Singh, 2000).

1.7.3 Cytokine profile analysis

Cytokines are soluble proteins that serve as messengers in the immune response. Cytokines also take part in cell communication, cell growth, differentiation as well as the formation and development of blood cells. Acting in autocrine, paracrine and endocrine manners, cytokines have been attributed to various disorders including cancer, autoimmune disorders and septic shock. There are also possible involvements of cytokines in many other diseases.

Pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α are biomarkers of inflammation. TNF-α have been documented to have pro-inflammatory effects on various diseases including rheumatoid arthritis, psoriasis, inflammatory bowel disease and sarcoidosis (Glaudemans *et al.*, 2010; Sanchez-Muñoz *et al.*, 2008). Some cytokines are also associated with the pathogenesis and progression of heart failure (Oikonomou *et al.*, 2011). On the other hand, anti-inflammatory cytokine such as IL-4 inhibits IFN-γ activation while IL-10 inhibits the production of IL-12, other pro-inflammatory cytokines as well as IL-17E (Zhou *et al.*, 2010). Cytokine profile analysis is important as a biomarker for various disorders. Many techniques can be used to measure the levels of cytokines such as bioassay, immunoassay and flow cytometry. Bioassay is one of the common techniques used. Bioassay measures the potency of substance to achieve an intended biological effect (Meager, 2006). Although it has appropriate sensitivity and the ability to measure biologically active molecules, it has several downsides such as a long turnaround time, poor precision and poor specificity (Burtis *et al.*, 2012).

Another method employed to measure cytokines levels is immunoassays. One of the most commonly used immunoassay technique is enzyme-linked immunosorbent assay (ELISA). Since commercially-available ELISA kits can detect single cytokine at a time, a multiplexed system has been introduced to solve this problem. Multiplex is highly sensitive and allows the detection up to 100 or more cytokines in a single microplate well. Nevertheless, it often measures total cytokine concentration which normally includes the non-functional molecules as well (Remick, 2006).

Flow cytometry is a rapid detection of intracellular cytokine detection. It can detect cytokines in blood and other body fluids including cerebrospinal and synovial

fluids. The disadvantage of using flow cytometry is the fact that identification and quantification of cytokines is complex and needs proper skill. Another drawback is the appearance of background induced autofluorescense in some body fluids (Burtis *et al.*, 2012).

1.8 Problem statement and justification of the study

Inflammatory diseases are globally identified causes of morbidity among the general population (Dewanjee *et al.*, 2013). Inflammation possesses a pivotal role in respiratory diseases. It leads to airway dysfunction and tissue remodelling. Thus, it is considered as a primary target for effective treatments. However, although current drugs available in the market such as glucocorticoid drugs are effective in treating asthma, they are less effective in treating chronic obstructive pulmonary disease. Besides, they also demonstrate side effects (Becker *et al.*, 2006) such as impaired growth in children, decreased bone mineral density, skin thinning and bruising and cataracts (Dahl, 2006). Hence, there is an increase demand for the development of new treatment modalities to treat lung inflammation.

In contrast to the side effects exhibited by the commercial anti-inflammatory drugs, alternative remedies based on natural products have been reported to have better pharmacological profile with lower toxicities (Patil and Patil, 2017). Besides their biocompatibility, natural products are cheaper alternatives for the treatment of inflammatory diseases (Patil and Patil, 2017). Therefore, natural products are sensible candidates in novel drug discovery.

According to the World Health Organization, nearly 50% of the population in China rely on traditional Chinese medicine as the main healthcare treatment modality (Nadimpalli, 2017). Similarly, the majority of population (80%) in Africa relies on traditional, complementary and alternative medicine (Nadimpalli, 2017). Moreover,

12.5% of the British population has been reported to have used complementary and alternative medicine (Shankar, 2014) in their lifetimes for at least once. Nevertheless, the popularity of alternative therapies has raised some concerns due to the lack of evidence-based literature on the effectiveness and safety of the natural product, indicating that more studies on natural product should be conducted. Hence, this study is conducted to provide a better insight on *L. rhinocerus*.

1.9 Objectives of the study

The aim of the study is to investigate the effects of various *L. rhinocerus* extracts on BEAS-2B. The specific objectives of the study are:

1. To perform and identify the best extraction methods and solvents for the extractions of *L. rhinocerus*.
2. To quantify the amount of linoleic acid from the *L. rhinocerus* extracts using HPLC.
3. To quantify the amount of β-glucan from the *L. rhinocerus* extracts using assay kit.
4. To determine the cytotoxic effect and apoptotic activities of *L. rhinocerus* extracts on BEAS-2B cell line.
5. To determine the effect of various *L. rhinocerus* extracts on cytokine profile of BEAS-2B cell line.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

The experimental study was conducted to understand the effect of administering *L. rhinocerus* extracts on BEAS-2B cell line. In order to investigate the study hypothesis, this research was designed to incorporate the following methods that were important to provide answers to the hypothesis including 1) HPLC method for the detection and quantification of linoleic acid in *L. rhinocerus* extracts, 2) quantification of β-glucan levels using Yeast & Mushroom β-Glucan Assay Kit (Megazyme International Limited, Bray, Ireland), 3) MTS assay for the proliferative effect of *L. rhinocerus* extracts on BEAS-2B lung cell line, 4) apoptosis assay using a flow cytometry for the determination of apoptotic effect of *L. rhinocerus* and 5) cytokine profile analysis for the determination of *L. rhinocerus* effects on BEAS-2B cytokine profile. HPLC-related experiments were carried out in Chromatography Laboratory, Institute for Research in Molecular Medicine while cell culture-related experiments were performed at the Craniofacial Science Laboratory, School of Dental Sciences, Culture Laboratory, School of Health Sciences, Universiti Sains Malaysia, Kelantan.

2.2 Materials

2.2.1 Chemicals, reagents and analytical kits

All chemicals, reagents, and analytical kits used in this study are as listed in Tables 2.1 and 2.2 respectively.

Table 2.1: List of chemicals and reagents

Item	Manufacturer/Supplier
4-hydroxy-3-methoxyphenylacetic acid	Sigma Chemicals (St Louis, USA)
Acetic acid (analytical grade)	Thermofisher (Massachusetts, USA)
Acetonitrile (HPLC grade)	Merck (Darmstadt, Germany)
Benzoic acid	Sigma Chemicals (St Louis, USA)
Caffeic acid	Sigma Chemicals (St Louis, USA)
Carminic acid	Sigma Chemicals (St Louis, USA)
Chlorogenic acid	Sigma Chemicals (St Louis, USA)
Dimethyl sulphoxide	Bio basic (Markham, Canada)
DL-2-2-aminobutyric acid	Sigma Chemicals (St Louis, USA)
Dulbecco's Modified Eagle's medium	Gibco (Massachusetts, USA)
Ethanol	Merck (Darmstadt, Germany)
Foetal Bovine Serum	Gibco (Massachusetts, USA)
Frusemide	Sigma Chemicals (St Louis, USA)
Hexane (Analytical grade)	Merck (Darmstadt, Germany)
Hexane (HPLC grade)	Merck (Darmstadt, Germany)
Hydrocortisone	Sigma Chemicals (St Louis, USA)
Ibuprofen	Sigma Chemicals (St Louis, USA)
Indomethacin	Sigma Chemicals (St Louis, USA)
L-Ascorbic acid	Sigma Chemicals (St Louis, USA)
Linoleic acid (Appendix D)	Acros Organics (New Jersey, USA)
Mephenesin	Sigma Chemicals (St Louis, USA)

Table 2.1: (continued)

Item	Manufacturer/Supplier
Methanol (HPLC grade)	Merck (Darmstadt, Germany)
Niacinamide	Sigma Chemicals (St Louis, USA)
p-coumaric acid	Sigma Chemicals (St Louis, USA)
Penicillin-Streptomycin	Gibco (Massachusetts, USA)
Petroleum ether	Merck (Darmstadt, Germany)
Phosphate-buffered saline	Amresco (Ohio, USA)
Prednisolone	Sigma Chemicals (St Louis, USA)
Trans-cinnamic acid	Sigma Chemicals (St Louis, USA)
Trypan blue	Sigma (Deisenhofen, Germany)
Trypsin TrypLE Express	Gibco (Scotland, United Kingdom)

Table 2.2: List of analytical kits

Item	Manufacturer/Supplier
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay	Promega (Wisconsin, USA)
FITC Annexin V Apoptosis Detection Kit I	Becton Dickinson (New Jersey, USA)
Human T-helper 1/T-helper 2/T-helper 17 Cytokines Multi-Analyte ELISArray Kit	Qiagen (Hilden, Germany)
Yeast & Mushroom β-Glucan Assay Kit	Megazyme, International Limited (Bray, Ireland)

N/B: FITC = fluorescein isothiocyanate.

2.2.2 Laboratory equipment, apparatus, computer software and applications

All laboratory equipment, computer software and applications used in this study are as listed in Tables 2.3 and 2.4.

2.2.3 *L. rhinocerus* mushroom

L. rhinocerus was obtained in cultivated dried powdered form from Ligno Biotech Sdn. Bhd (Selangor, Malaysia). (Appendix C).

2.2.4 Cell culture

BEAS-2B was purchased from American Type Culture Collection (Manassas, USA) (resource number: ATCC® CRL 9609™).

2.2.5 Medium

2.2.5(a) Complete Dulbecco's Modified Eagle's Medium

Complete Dulbecco's Modified Eagle's Medium was prepared by adding 1% penicillin-streptomycin and 10% foetal bovine serum. The complete Dulbecco's Modified Eagle's Medium was kept at 4^{o}C. The medium was pre-warmed at 37^{o}C prior to use.

2.2.6 Solution

2.2.6(a) Ethanol 70%

Absolute ethanol (70 ml) was mixed with 30 ml of distilled water to prepare a 70% ethanol solution.

Table 2.3: List of laboratory equipment

Item	Manufacturer/Supplier
Autoclave	Hirayama (Saitama, Japan)
Biosafety cabinet class II	NuAire (Minnesota, USA)
Centrifuge	Eppendorf (Hamburg, Germany)
Carbon dioxide incubator	NuAire (Minnesota, USA)
ELISA plate reader	SunRise (Tecan, Austria)
Freeze dryer	Fisher Scientific (Pittsburgh, USA)
Fume hood	NuAire (Minnesota, USA)
Haemocytometer	Laboroptik (Lancing, UK)
HPLC	Agilent (California, USA)
Light microscope	ZEISS (Oberkochen, Germany)
Microliter syringe	Hamilton (Nevada, USA)
Multichannel pipette	Eppendorf (Hamburg, Germany)
Pipette	Eppendorf (Hamburg, Germany)
Roller mixer	Stuart (Staffordshire, UK)
Rotary evaporator	Buchi (Flawil, Switzerland)
Serological pipette	Eppendorf (Hamburg, Germany)
Soxhlet extraction set	Pyrex (Pennsylvania, USA)
Vacuum filter set	Sartorius (Göttingen, Germany)
Water bath	Fisher Scientific (Pittsburgh, USA)
Water bath sonicator	Fisher Scientific (Pittsburgh, USA)

Table 2.4: List of computer software and applications

Item	Manufacturer/Supplier
Microsoft Office 2007	Microsoft Corp. (Washington, USA)
OpenLAB 2.1	Agilent (California, USA)
Statistical Programme for Social Sciences (SPSS), version 20	SPSS Inc. (Illinois, USA)

2.3 Methodology

2.3.1 HPLC determination of linoleic acid in *L. rhinocerus* extracts

Natural products used as traditional remedies offer a wide variety of substances for various diseases. However, only a few active constituents may contribute to their effects which are important bioactive compounds. The fact that natural products contain a wide range of substances complicates the identification and characterization processes. Therefore, a sensitive and specific detection method is crucial for proper identification of bioactive compounds. In addition, non-sophisticated and readily accessible equipment are preferable. In this chapter, a new method is developed to confirm the presence of linoleic acid in *L. rhinocerus* extracts and for its quantification.

2.3.1(a) Extraction methods

Soxhlet, maceration and ultrasound-assisted extraction methods were investigated for extraction of fatty acids specifically linoleic acid from *L. rhinocerus* powder. Figure 2.1 summarises the extraction method.

2.3.1(a)(i) Soxhlet extraction

L. rhinocerus powder (50 g) was weighed and transferred into an extraction thimble (Figure 2.2). Then, 500 ml of distilled water was transferred into a round bottom flask. The soxhlet extraction set was set up as in Figure 2.2 and was allowed to run for eight hours per day for five consecutive days. The extract was then concentrated using a rotating evaporator (Buchi, Flawil, Switzerland). The concentrated extract was dried using a freeze dryer before being weighed and stored in a tightly capped container for further analysis.

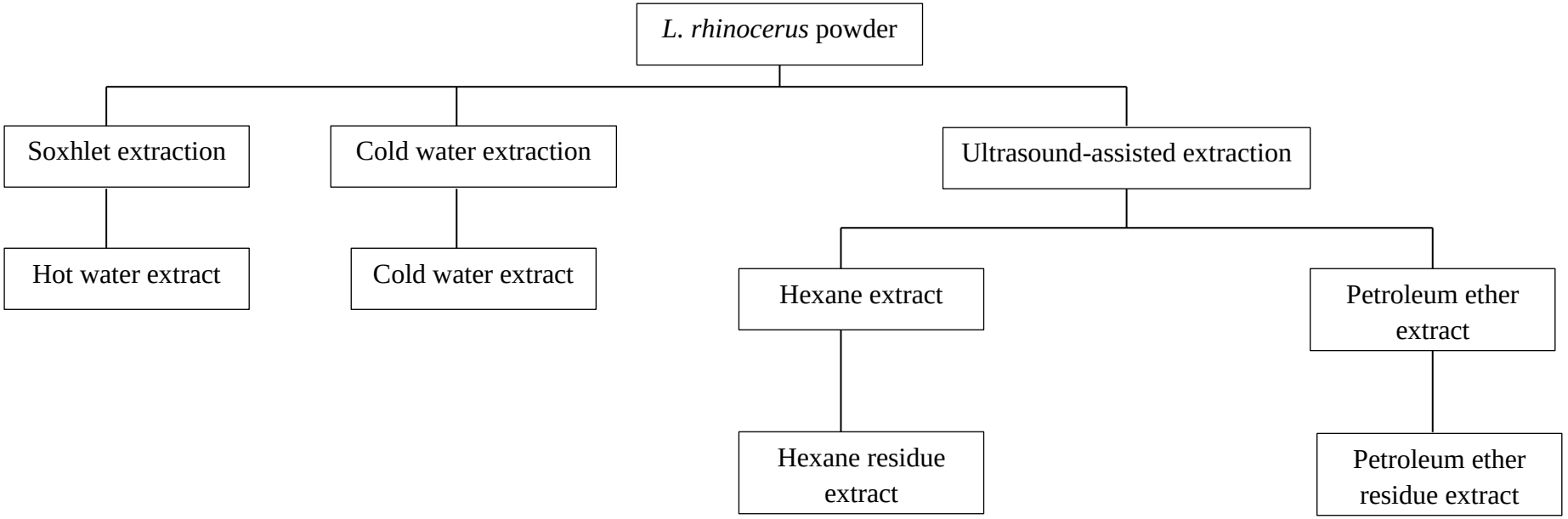

Figure 2.1: Summary of extraction methods

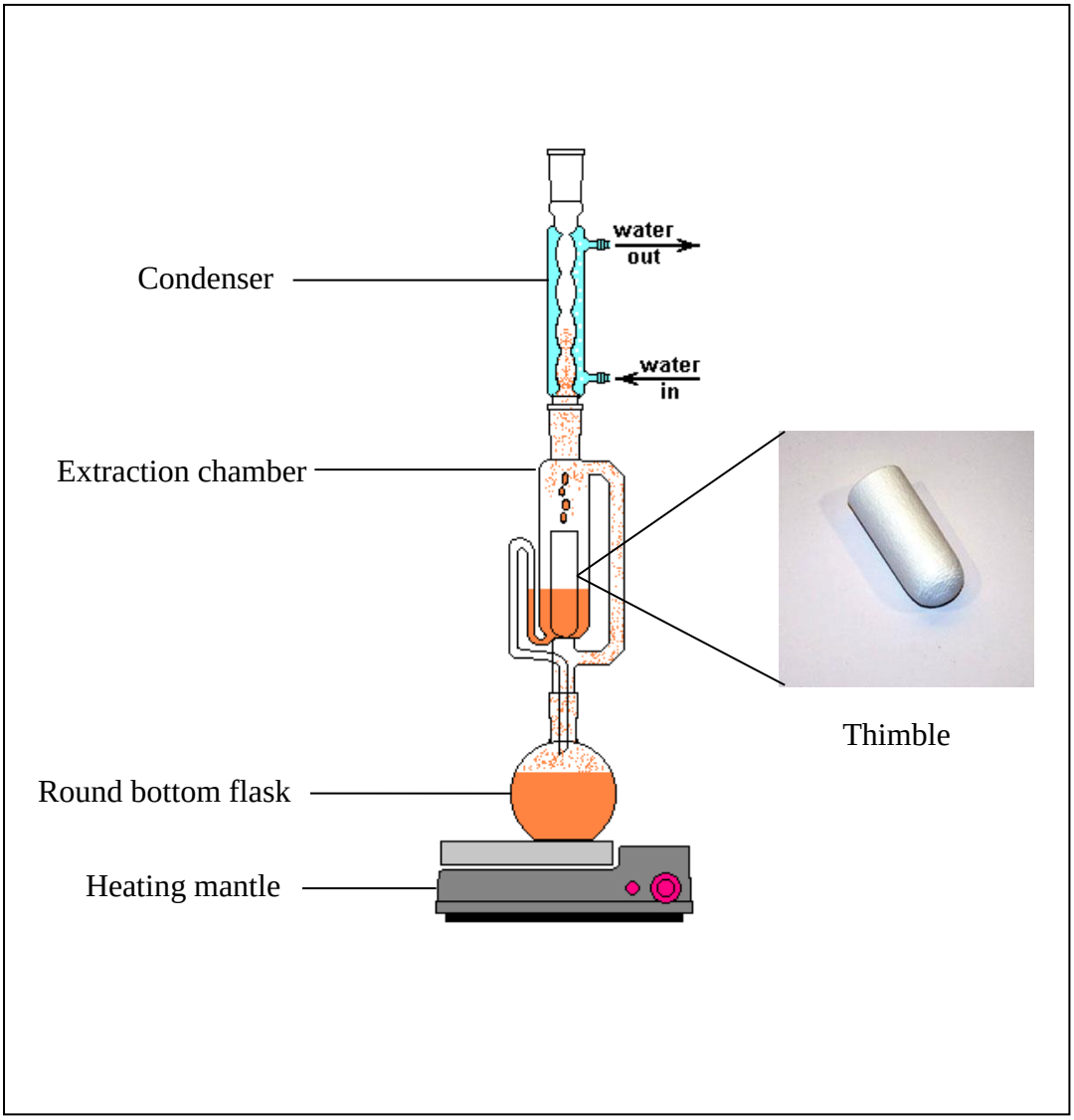

Figure 2.2: Schematic representation of a soxhlet extraction set. (Adapted from: *https://de.wikipedia.org/wiki/Datei:Soxhlet_mechanism.gif*)

2.3.1(a)(ii) Cold water extraction

L. rhinocerus powder (50 g) was macerated in 500 ml of water for 24 hours in cold room (4^{o}C) with agitation. After 24 hours, the extract was centrifuged at 2500 x g for 10 min. Then, the supernatant was dried. The extract was weighed before being stored in a tightly closed container.

2.3.1(a)(iii) Ultrasound-assisted extraction

Petroleum ether and hexane were used as solvents in this extraction method. *L. rhinocerus* powder (50 mg) was first dissolved in 500 ml of either solvent and was transferred into a water bath sonicator for 30 minutes at 40^{o}C. The solution was centrifuged at 2500 x g for 10 minutes. Then, the supernatant was concentrated, dried and stored. The pellet was resuspended in 500 ml of water before being subjected to ultrasound in a water bath sonicator for another 30 minutes prior to centrifugation (2500 x g for 10 minutes). The supernatant was collected, concentrated, dried, weighed and stored while the residues were further extracted with distilled water using similar technique.

2.3.1(b) Standard, internal standard and internal standard candidates

The standard, internal standard and internal standard candidates used in this analysis were as listed in Table 2.5.

2.3.1(c) Stock and working solutions

Stock solutions of linoleic acid (1 mg/ml) were prepared by dissolving 5 mg of linoleic acid in 5 ml of methanol followed by storage at -20^{o}C. The linoleic acid concentrations chosen for the calibration curve were 5, 15, 25, 50, 100 and 200 µg/ml. The working concentrations were prepared fresh daily by making further dilution of prepared stock solutions in methanol.

Table 2.5 List of standard, internal standard and internal standard candidates

Type	Item
Standard	Linoleic acid
Internal standard	Indomethacin
Internal standard candidates	Hydrocortisone
	Mephenesin
	Prednisolone
	Niacinamide
	Benzoic acid
	Carminic acid
	Chlorogenic acid
	p-coumaric acid
	4-hydroxy-3-methoxyphenylacetic acid
	Caffeic acid
	Ibuprofen
	L-ascorbic acid
	Trans-cinnamic acid
	Frusemide
	DL-2-2-aminobutyric acid

A stock solution of the internal standard (1 mg/ml) was prepared by dissolving 5 mg of indomethacin in 5 ml of methanol before storage at -20^{o}C. A working standard solution of 40 µg/ml in methanol was prepared fresh daily by taking 40 µl of the stock solution and diluting in 960 µl of methanol.

2.3.1(d) Method optimisation

The method optimisation in this study is based on the method described by Guarrasi *et al.* (2010) with some slight modification. Reverse phase HPLC experiment was performed on an Agilent 1260 Infinity System (Agilent Technologies, Santa Clara, USA). Instrument control and data collection were carried out using an Agilent OpenLAB 2.1 software. A COSMOSIL 5C18-MS-II (250 X 4.6 mm I.D: 5 µm) and guard column COSMOSIL 5C18-MS-II guard column (10 X 4.6 mm I.D: 5 µm) were obtained from Nacalai Tesque, Inc, Japan. The flow rate for the analysis was 1 ml/min. The injection volume was 10 µl, while ultraviolet detection wavelength was set at 208 nm and 220 nm. A C_{18} column was used. Three combinations of mobile phase were tested 1) acetonitrile and water (90:10), 2) acetonitrile and methanol (90:10) and acetonitrile, methanol and hexane (90:8:2). The effect of 0.2% organic modifier was also tested. The mobile phase was premix prior to analysis. The summary of chromatographic condition is as shown in Table 2.6.

Table 2.6: Chromatographic equipment, parameters and mobile phase

Pump	Infinity quaternary pump 1260 (Agilent, USA)
Detector	Infinity Diode Array Detector 1260 (Agilent, USA)
Controller	IBM computer equipped with OpenLAB 2.1 software (Agilent, USA)
Analytical column	COSMOSIL 5C18-MS-II (250 X 4.6 mm I.D: 5 µm) (Nacalai Tesque, Inc, Japan).
Guard column	COSMOSIL 5C18-MS-II guard column (10 X 4.6 mm I.D: 5 µm) (Nacalai Tesque, Inc, Japan).
Mobile phase	A mixture of acetonitrile, methanol and hexane (acidified with acetic acid; refer to text for detail of optimization and final composition).
Flow rate *	1.00 ml/min
Injection volume	10 µl
Analysis time	The total analytical time was approximately 15 minutes

***** The mobile phase which consisted of acetonitrile:methanol:hexane (90:8:2) was prepared fresh daily. Acetic acid (0.2%) was added into the mixture followed by filtration using a polytetrafluoroethylene membrane filter (0.45 µm) (Sartorius, Germany).

2.3.1(e) Validation

The following validation parameters were determined: linearity, precision and accuracy. To validate the linearity of the method, calibration graphs were constructed in the range of 5 to 200 μg/ml for three days. Linearity of the calibration graph was determined by the correlation coefficient obtained for the regression line. For precision and accuracy, a set of quality controls was also performed at four concentrations (80, 90, 110 and 150 μg/ml) daily for three consecutive days. To measure the accuracy of the method, the quality control values should be within 15% of their respective nominal value except at lower limit of quantification, where it should not deviate by more than 20% (Food and Administration, 2015). The precision is determined by the coefficient of variation. The coefficient of variation at each concentration level should not exceed 15% except for the lower limit of quantification, where it should not exceed 20% (Food and Administration, 2015). The preparation of linoleic acid standard solutions for validation is shown in Table 2.7.

2.3.1(f) Measurement of linoleic acid in *L. rhinocerus* extracts

The samples were prepared by dissolving *L. rhinocerus* in methanol. Water-based extract such as hot water, cold water, hexane residue and petroleum ether residue extracts were prepared at 10 mg/ml while the concentration for solvent-based extracts such as hexane and petroleum ether extracts were at 100 μg/ml. A set of calibrators and quality controls (Table 2.8) were also included during the measurement to ensure the linearity, precision and accuracy of the method.

Table 2.7: Preparation of linoleic acid standard solutions for validation

Concentration (µg/ml)	Volume (µl)		
	Methanol	Linoleic acid	Indomethacin
Calibrators			
5	955	5	40
15	945	15	40
25	935	25	40
50	910	50	40
100	860	100	40
200	760	200	40
Quality controls			
80	880	80	40
90	870	90	40
110	850	110	40
150	810	150	40

Table 2.8: Preparation of calibrators and quality controls for linoleic acid measurement

Concentration (µg/ml)	Volume (µl)		
	Methanol	**Linoleic acid**	**Indomethacin**
Calibrators			
5	955	5	40
25	935	25	40
50	910	50	40
100	860	100	40
200	760	200	40
Quality controls			
15	945	15	40
80	880	80	40
150	810	150	40

2.3.2 β-glucan quantification

Quantification of β-glucan in *L. rhinocerus* extracts was conducted using Mushroom and Yeast Beta-Glucan Assay Kits (Megazyme. International Limited, Bray, Ireland). The total and alpha glucans were measured. β-glucan was determined by subtracting the alpha from the total glucans values.

2.3.3 Determination of the effect of *L. rhinocerus* on BEAS-2B

Evaluation of the effect of *L. rhinocerus* effects on BEAS-2B was performed using three analyses. The first analysis is the proliferation assay. The assay was done using an MTS assay kit, a colourimetric assay that measures the metabolic activity of a population of cells. Proliferation assay is important to investigate the effects of biological components in the extracts and to assess their cytotoxicity since the extracts contain a wide range of secondary metabolites, some of which could be cytotoxic. The second analysis is the determination of apoptotic effect of *L. rhinocerus* on BEAS-2B using an FITC Annexin V Apoptosis Kit. FITC Annexin V staining precedes the loss of membrane integrity which accompanies the final stages of cell death resulting from either apoptotic or necrotic processes. The third analysis was to determine the effect of *L. rhinocerus* on BEAS-2B cytokines profile using Human T-helper 1/T-helper 2/T-helper 17 Cytokines Multi-Analyte ELISArray Kits (Qiagen, Hilden, Germany). This kit profiled the levels of T-helper 1, T-helper 2 and T-helper 17 cytokines using the conventional and simple ELISA techniques.

2.3.3(a) Aseptic techniques

Cell culture work was conducted in a biosafety cabinet which conferred protection against harmful particles. All cell culture-related work was carried out under sterile conditions with the practice of aseptic techniques.

2.3.3(b) Preparation of BEAS-2B cell line

Upon receipt, the frozen BEAS-2B cell line was thawed immediately. Storage of the cell in liquid nitrogen may affect its viability and is therefore not encouraged. The thawed cell was transferred into a 15 ml centrifuge tube containing pre-warmed media (5 ml). The cell suspension was centrifuged at 800 x g for 10 minutes. The pellet was harvested and re-suspended in a complete growth medium. The cells were cultured in complete Dulbecco Modified Eagle's Medium at 37^{o}C and 5% carbon dioxide humidified atmosphere. The medium was changed every 2 to 3 days.

2.3.3(c) BEAS-2B cell maintenance

The cells were monitored for possible contamination or deterioration. When the cells were confluent, the old medium was removed and the cells were rinsed with phosphate-buffered saline (5 ml) to remove any unwanted particles which might harm the cells. TripLE Express was used to detach the cells from the flask surface. Once the cells were detached from the flask surface, complete medium was added immediately to avoid the cells from shrinking. The cell suspension was centrifuged at 800 x g for 5 minutes. Then, the pellet was harvested and re-suspended in a complete growth medium and cultured in flask. For cryopreservation, approximately 1 x 10^6 – 1 x 10^7 cells/ml was transferred to the cryovials containing 5% dimethyl sulphoxide in foetal bovine serum. The vials were sealed and labelled according to the date and passage number. The vials were frozen gradually at 4, -20 and -70^{o}C overnight and subsequently transferred to a liquid nitrogen storage tank.

2.3.3(d) Determination of biological contamination

Bacterial contamination was determined by visual inspection within a few days upon seeding. Generally, the infected culture appears turbid. Yeast appeared as

ovoid or spherical in shape while the mold was observed as clumps of spores under a 100x magnification. Mycoplasma contamination was indicated by the deterioration and detachment of cells from the flask, decreased cell proliferation rate, reduction in saturation density and agglutination as seen in the suspension cultures.

2.3.3(e) Cell counting

The cell number and viability were determined by a trypan blue assay using a haemocytometer slide. The cell suspension and trypan blue dye (1:1) was prepared and transferred to the haemocytometer chamber. The slide was then viewed under an inverted microscope at 100x magnification. The cells within the four corners of the grid were counted. The total number of cells was determined as below:

$C = Av \times 2 \times 10^4$ cells/ml

where, C = Cell concentration; Av = Average number of cells; 2 = Dilution factor.

2.3.3(f) Cell proliferation assay

BEAS-2B cells were treated with six different extracts 1) hot water 2) cold water 3) hexane 4) petroleum ether 5) aqueous residue of hexane and 6) aqueous residue petroleum ether extracts. The working solution of all extracts was prepared fresh prior to each treatment. *L. rhinocerus* extracts (20 mg) was weighed and transferred to a containing 20 ml of complete growth medium with 0.5% dimethyl sulphoxide to obtain a final concentration of 1000 µg/ml. The solution of each extract was filtered and a serial dilution was performed to obtain 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml of extract.

The confluence cells were trypsinised and 2×10^3 cells per well were seeded in a 96-well plate. After 24 hours of incubation, each well was replaced with 100 µl of media containing various concentrations of the extract as stated above. The

samples were prepared in quintuplicate along with the two control groups consisting of growth medium alone and growth medium with 0.5% dimethyl sulphoxide. The wells containing the growth medium alone without the presence of cells were also included as blank. The plates were incubated for five days respectively. During the selective incubation periods (day 1, 2, 3, 4 and 5), MTS solution (20 µl) was added into each well.

After 4 hours of incubation in 5% carbon dioxide incubator, the absorbance of each solution was measured at 490 nm by an ELISA plate reader. The viability of each sample was calculated and a graph was constructed from a series of different concentrations.

The formula for cell viability;

$$\text{Cell viability (\%)} = \left[\frac{(OD_S - OD_B)}{(OD_C - OD_B)}\right] \times 100$$

OD_S = optical density reading for treated wells

OD_C = Mean optical density for untreated wells

OD_B = Mean of optical density for blank wells

2.3.3(g) Apoptosis assay

Apoptosis assay was carried out using a FITC Annexin V Apoptosis Detection Kit I (Becton Dickinson, New Jersey, USA). In this assay, BEAS-2B cells were treated with six different extracts (hot water, cold water, hexane, petroleum ether, aqueous residue of hexane and aqueous residue petroleum ether) at 62.5, 125.0 and 250.0 µg/ml based on proliferation study. The working solution of all extracts was freshly prepared prior to the treatment. A 2.5 mg *L. rhinocerus* powder was

weighed under sterile conditions and was transferred to a tube containing 20 ml of growth medium with 0.5% dimethyl sulphoxide to obtain a final concentration of 250 µg/ml. The solution of each extract was filtered and a serial dilution was performed to obtain the extracts at 62.5, 125.0 and 250.0 µg/ml.

Confluence cells were trypsinised and 5 x 10^5 cells per well were seeded in T-75 flask. After 24 hours of incubation, the solution in each flask was replaced with 10 ml of medium containing various extract concentrations as mentioned above. The plates were incubated for 24 hours. After 24 hours, the cells were harvested, washed twice with phosphate-buffered saline, resuspended in a binding buffer (1 ml), stained with annexin V FITC and propidium iodide followed by incubation for 15 minutes at room temperature using a flow cytometer. Since annexin V FITC and propidium iodide are light-sensitive, all staining procedures were performed without direct exposure of intense light and incubation was done in the dark. A set of unstained cells, positive control and single stains for annexin V FITC and propidium iodide were also included for the compensation purposes as per required by the flow cytometry protocol.

2.3.3(h) Cytokine profile analysis

Cytokine profile analysis was carried out using a Human T-helper 1/T-helper 2/T-helper 17 Cytokines Multi-Analyte ELISArray Kits (Qiagen, Hilden, Germany). In this assay, BEAS-2B cells were treated with six different extracts (hot water, cold water, hexane, petroleum ether, aqueous residue of hexane and aqueous residue petroleum ether) at 62.5 µg/ml. The working solution of all extracts was prepared fresh prior to the treatment. After 48 hours of incubation, the cell culture supernatants were added into the respective ELISArray plate that has been prepared

according to the manufacturer protocol. After a series of incubation and washing, detection antibodies were added accordingly into the ELISArray plate followed by incubation for 1 hour before the addition of Avidin-HRP into the wells. After another series of incubation and washing, Developmental Solution was added into the ELISA wells followed by a Stop Solution after 15 minutes of incubation. Finally, Stop Solution was added into all wells and the absorbance was read at 450 nm.

2.3.4 Statistical analysis

Statistical analyses were performed using the Statistical Package of Social Science software, version 21.0. A one-way ANOVA was used to analyse the findings from the proliferation assay while Kruskal-Wallis and Mann-Whitney procedures were used for apoptosis assay and cytokine profile analysis. All statistical analyses were performed at significance level of $p < 0.05$.

CHAPTER 3

RESULTS

3.1 HPLC

3.1.1 Internal standard candidates

From the various drugs tested for suitability as an internal standard, 4-hydroxy-3-methoxyphenylacetic, benzoic, caffeic, carminic, chlorogenic, L-ascorbic, p-coumaric and trans-cinnamic and DL-2-2-aminobutyric acid as well as frusemide, niacinamide, mephenesin, ibuprofen, prednisolone and hydrocortisone (Figures 3.1-3.15) did not appear in the chromatogram during the 30 min of analytical time. However, indomethacin showed a tall and sharp peak at 3.25min (Figure 3.16). Therefore, indomethacin was chosen as the internal standard of choice for the linoleic acid HPLC analysis.

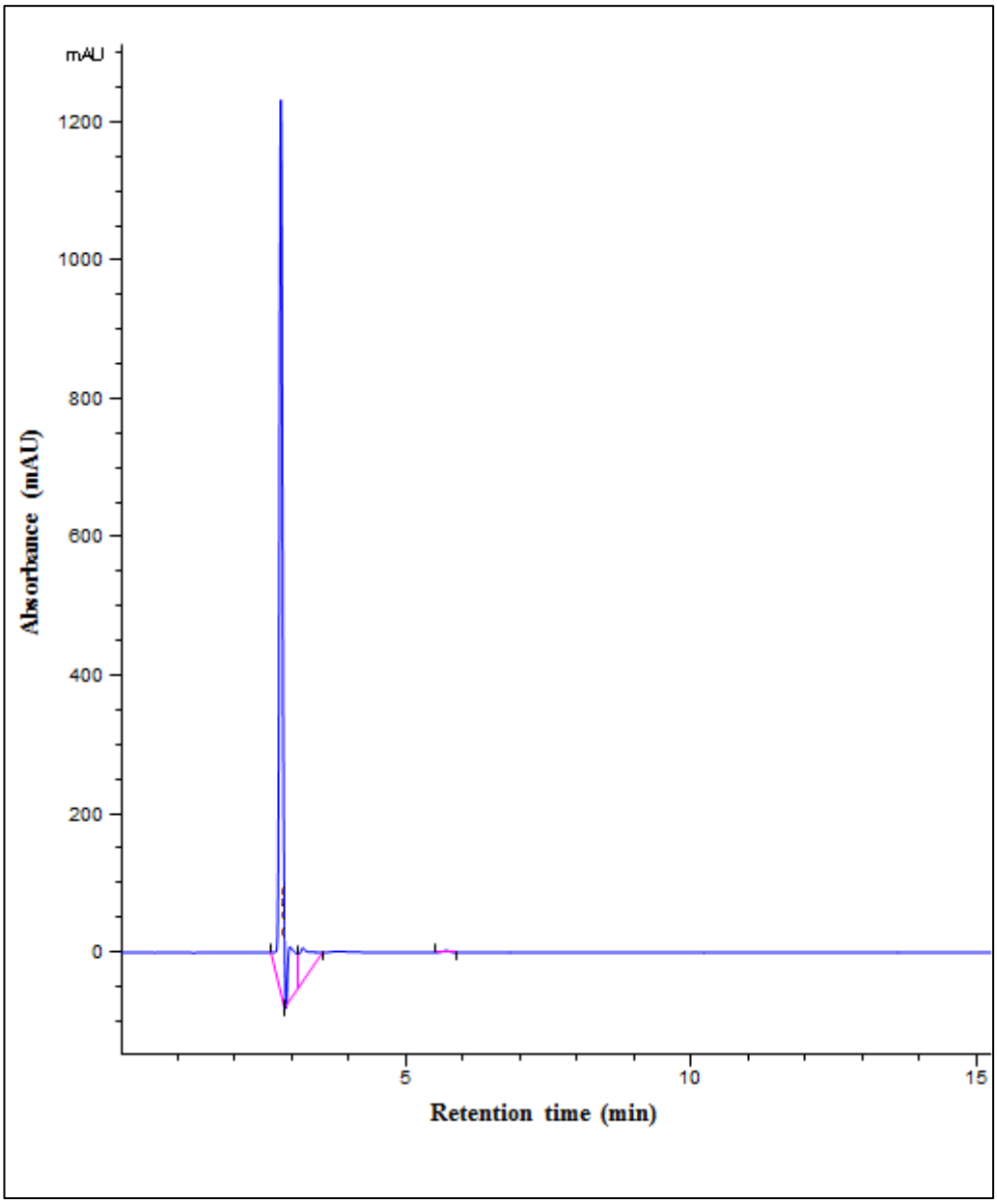

Figure 3.1: Chromatogram obtained after injection of 4-hydroxy-3-methoxyphenylacetic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

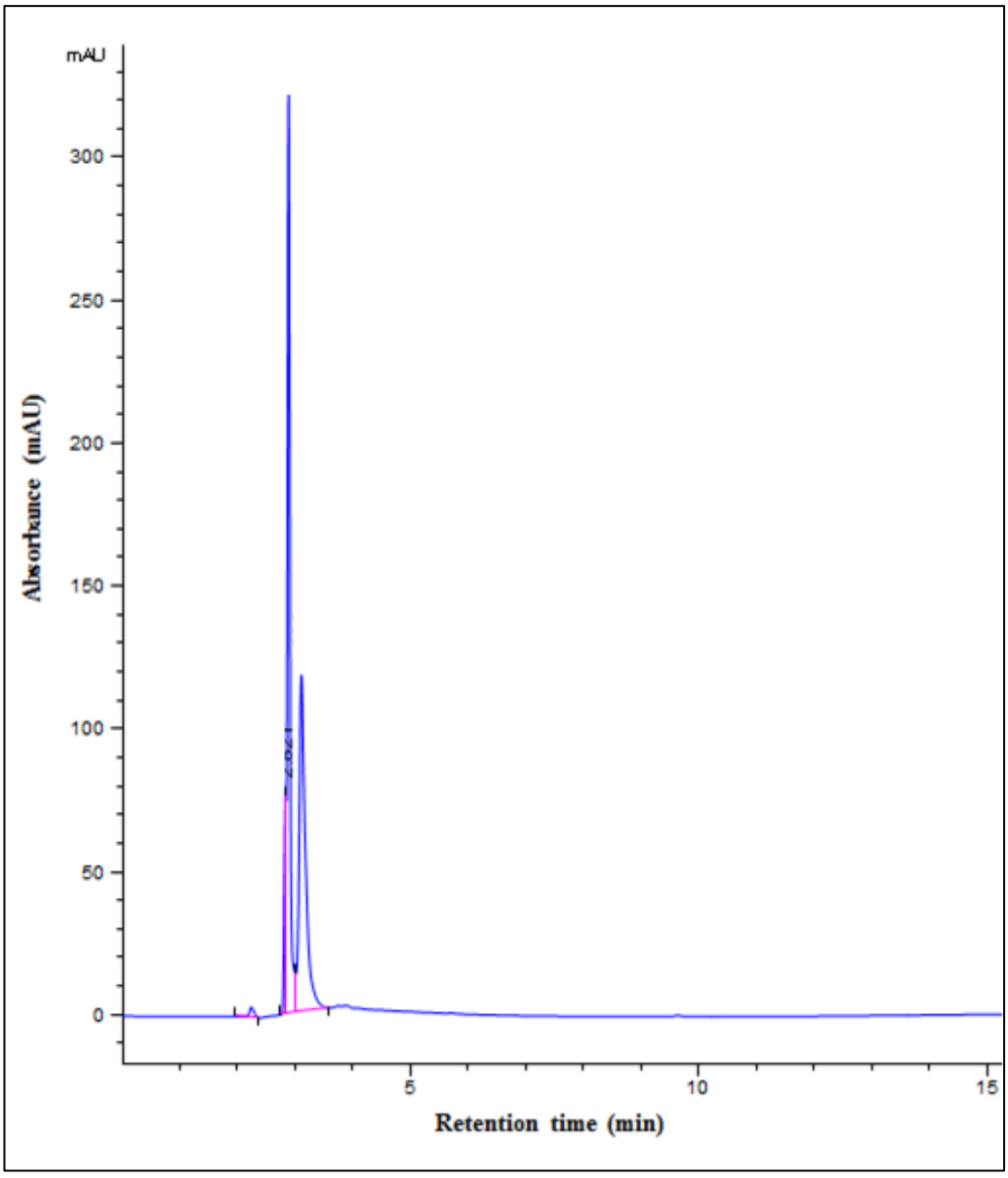

Figure 3.2: Chromatogram obtained after injection of benzoic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

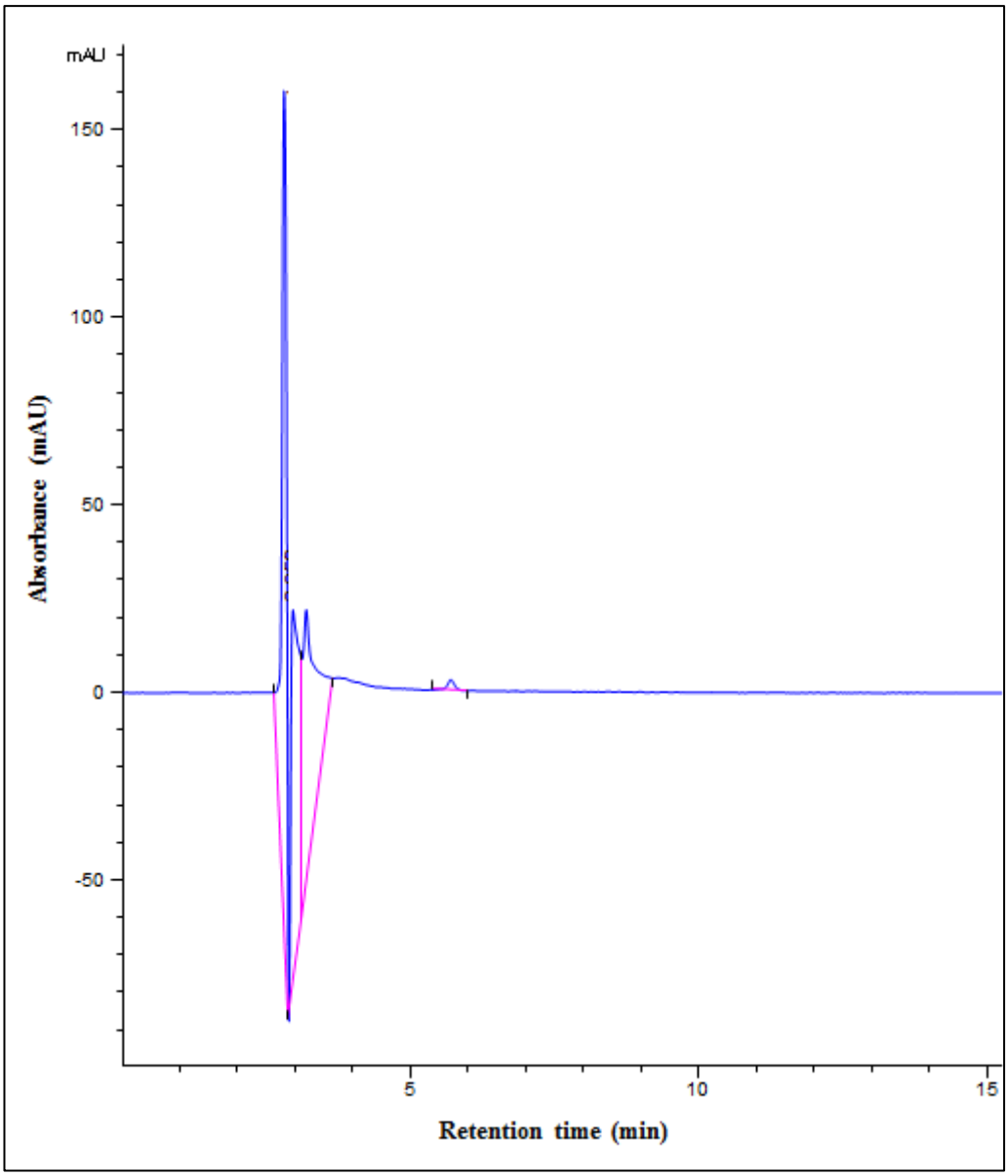

Figure 3.3: Chromatogram obtained after injection of caffeic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

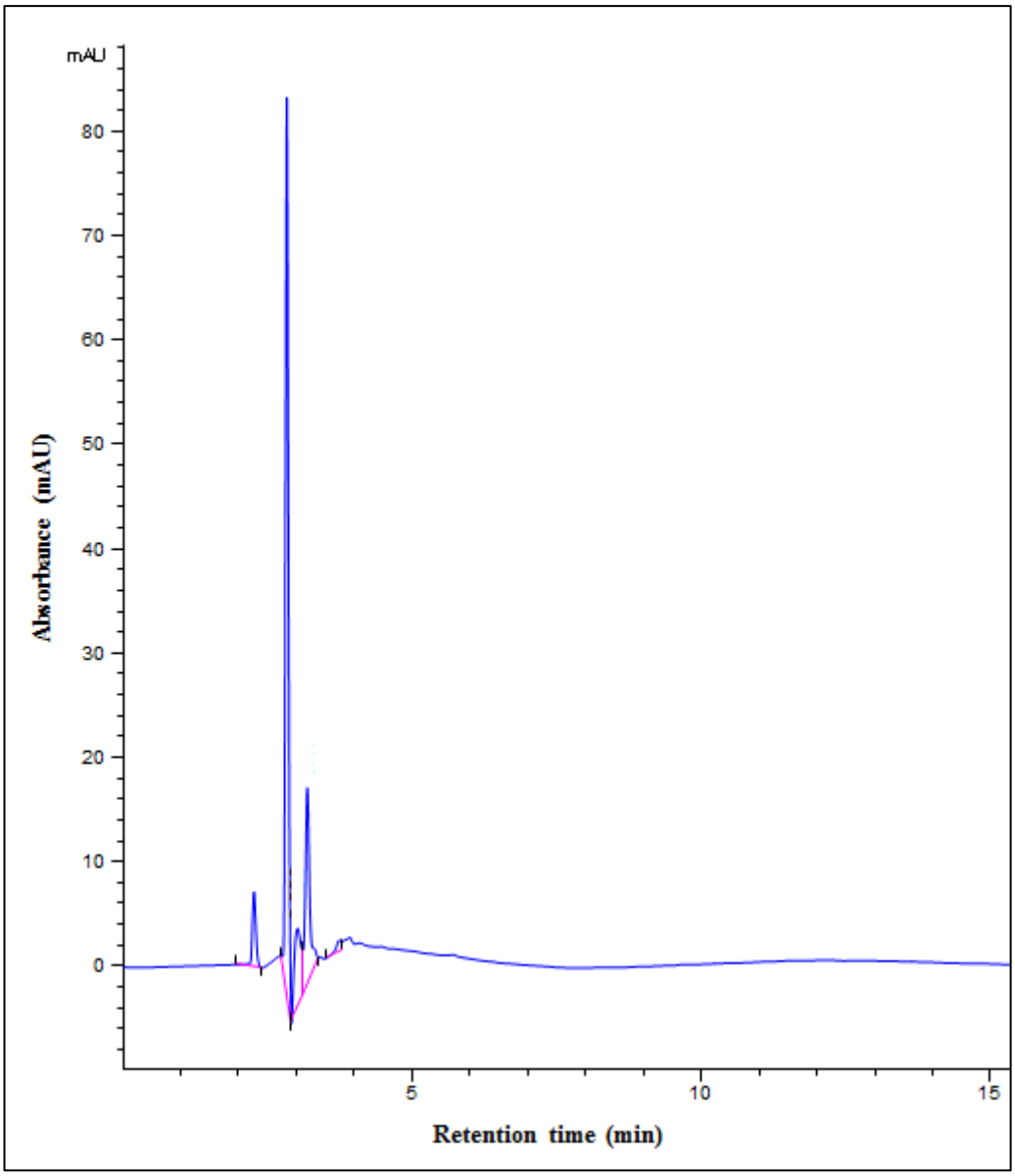

Figure 3.4: Chromatogram obtained after injection of carminic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

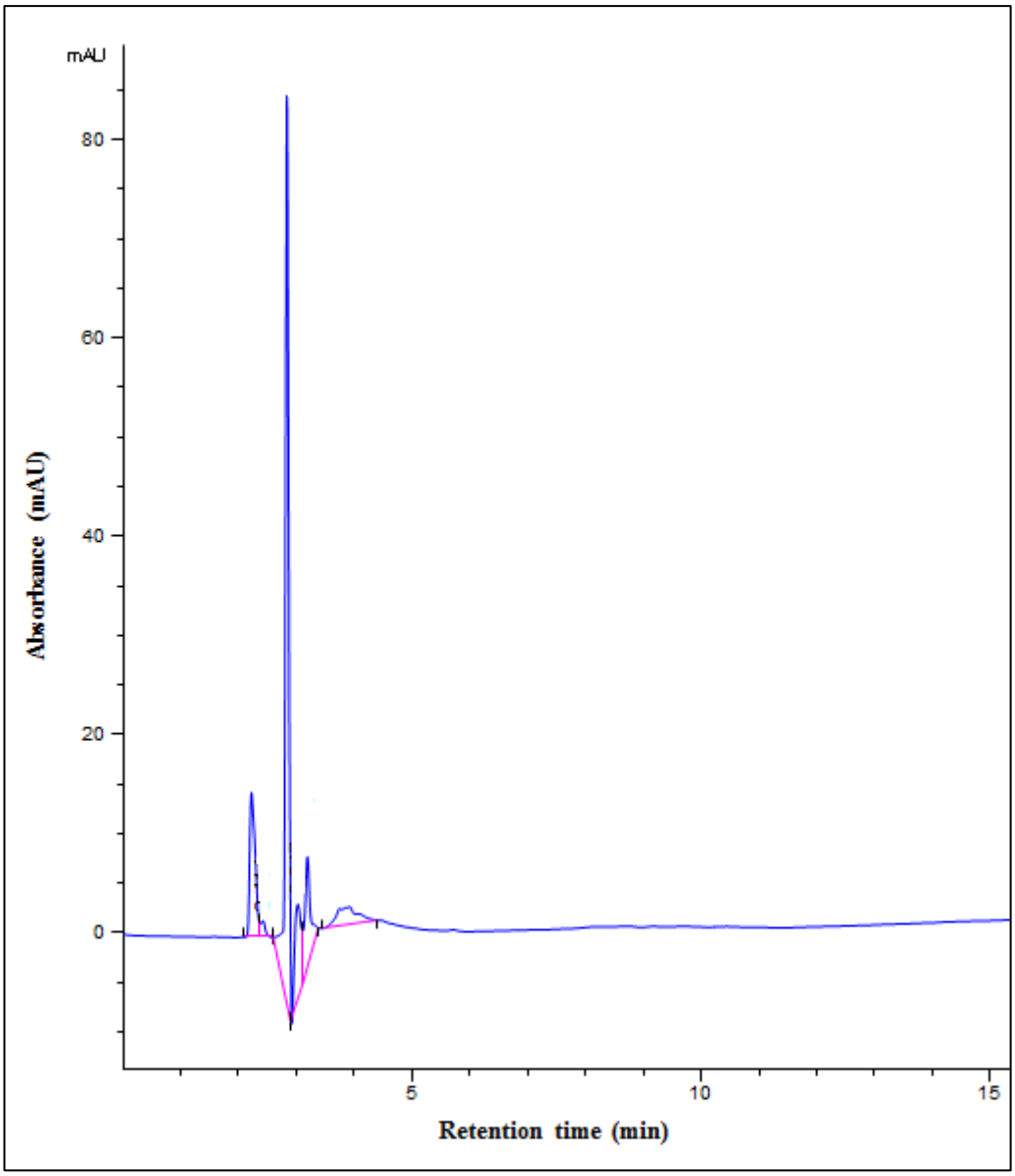

Figure 3.5: Chromatogram obtained after injection of chlorogenic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

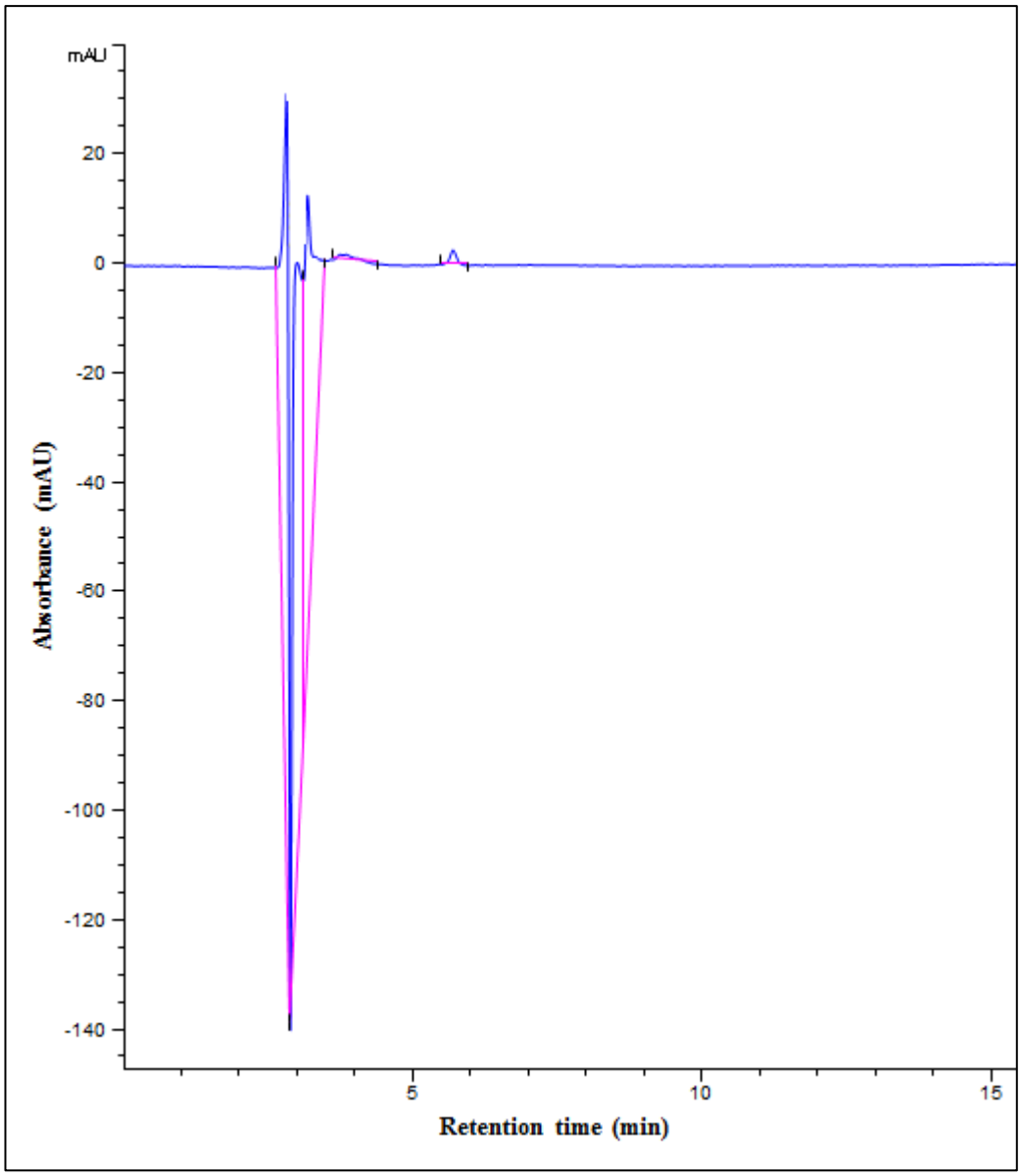

Figure 3.6: Chromatogram obtained after injection of L-ascorbic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

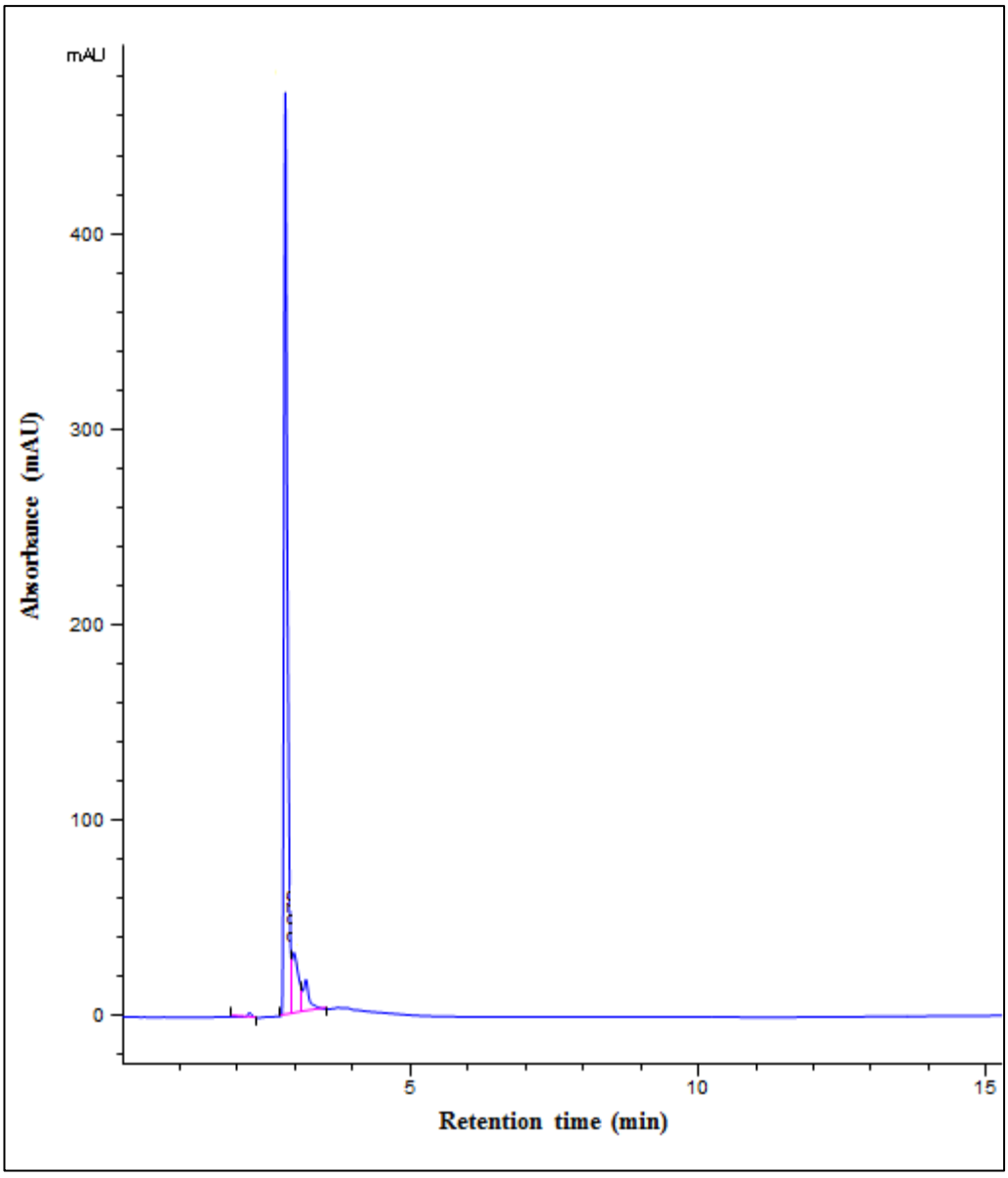

Figure 3.7: Chromatogram obtained after injection of p-coumaric acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

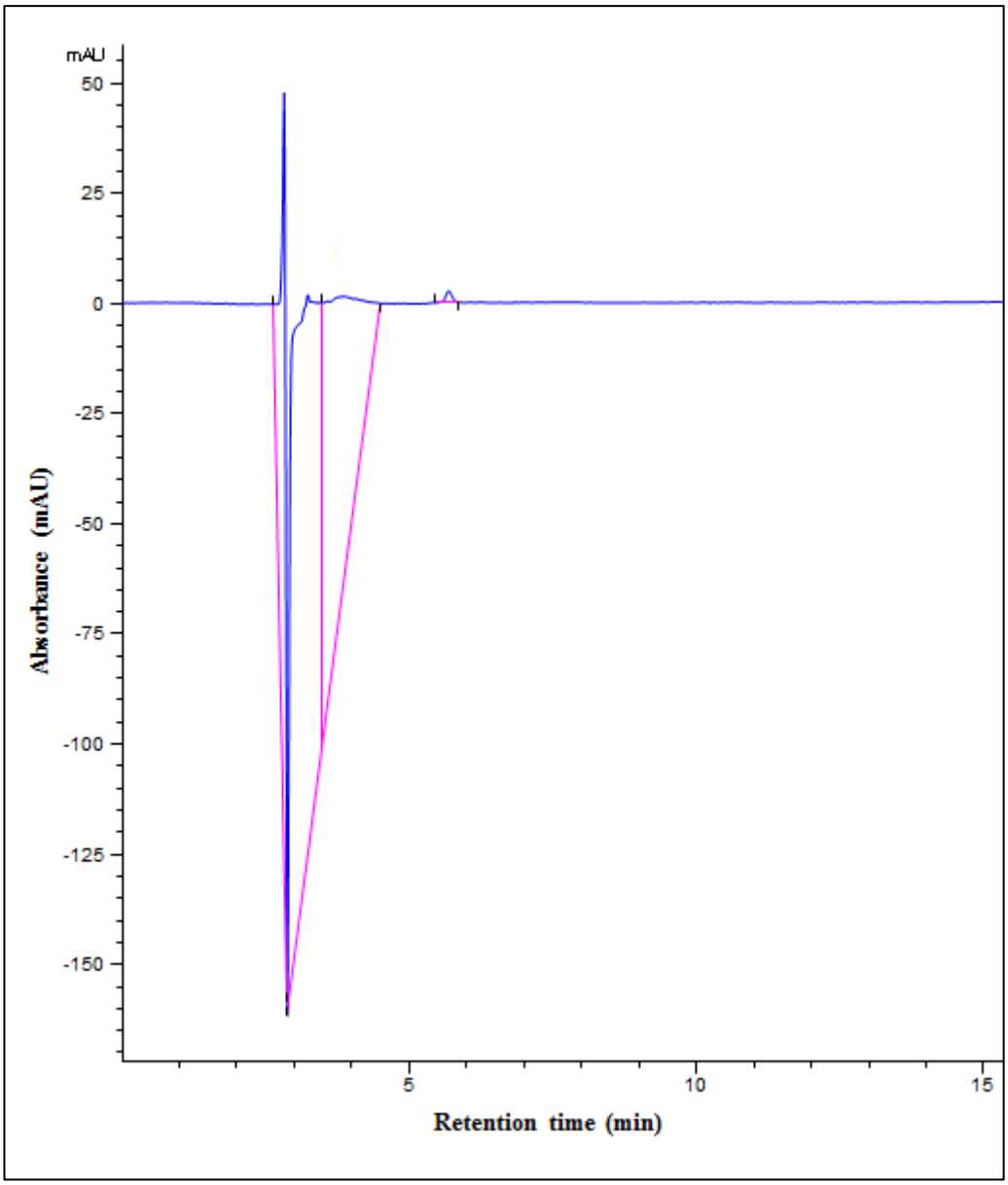

Figure 3.8: Chromatogram obtained after injection of trans-cinnamic acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

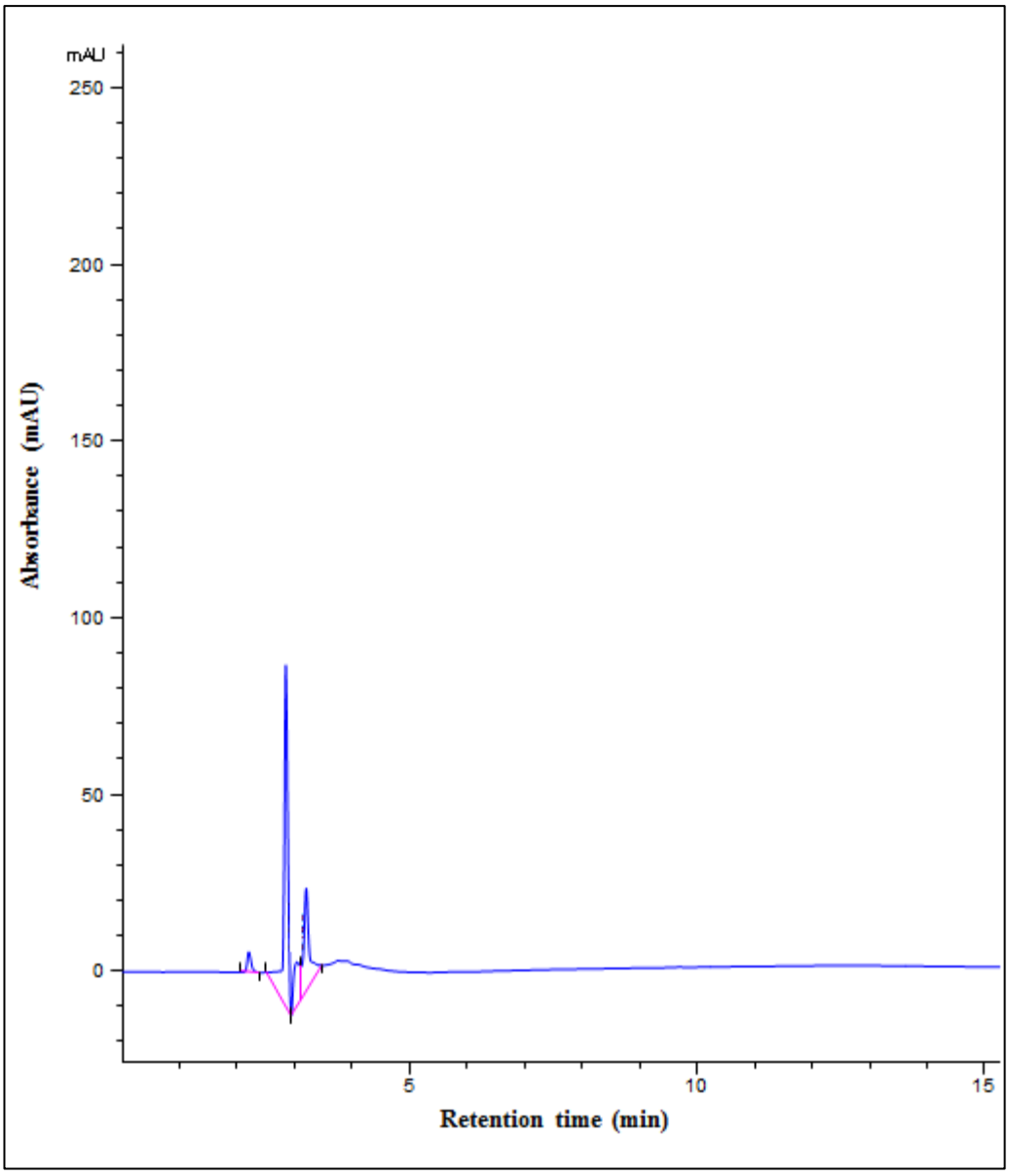

Figure 3.9: Chromatogram obtained after injection of DL-2-2-aminobutyric acid in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B**: Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

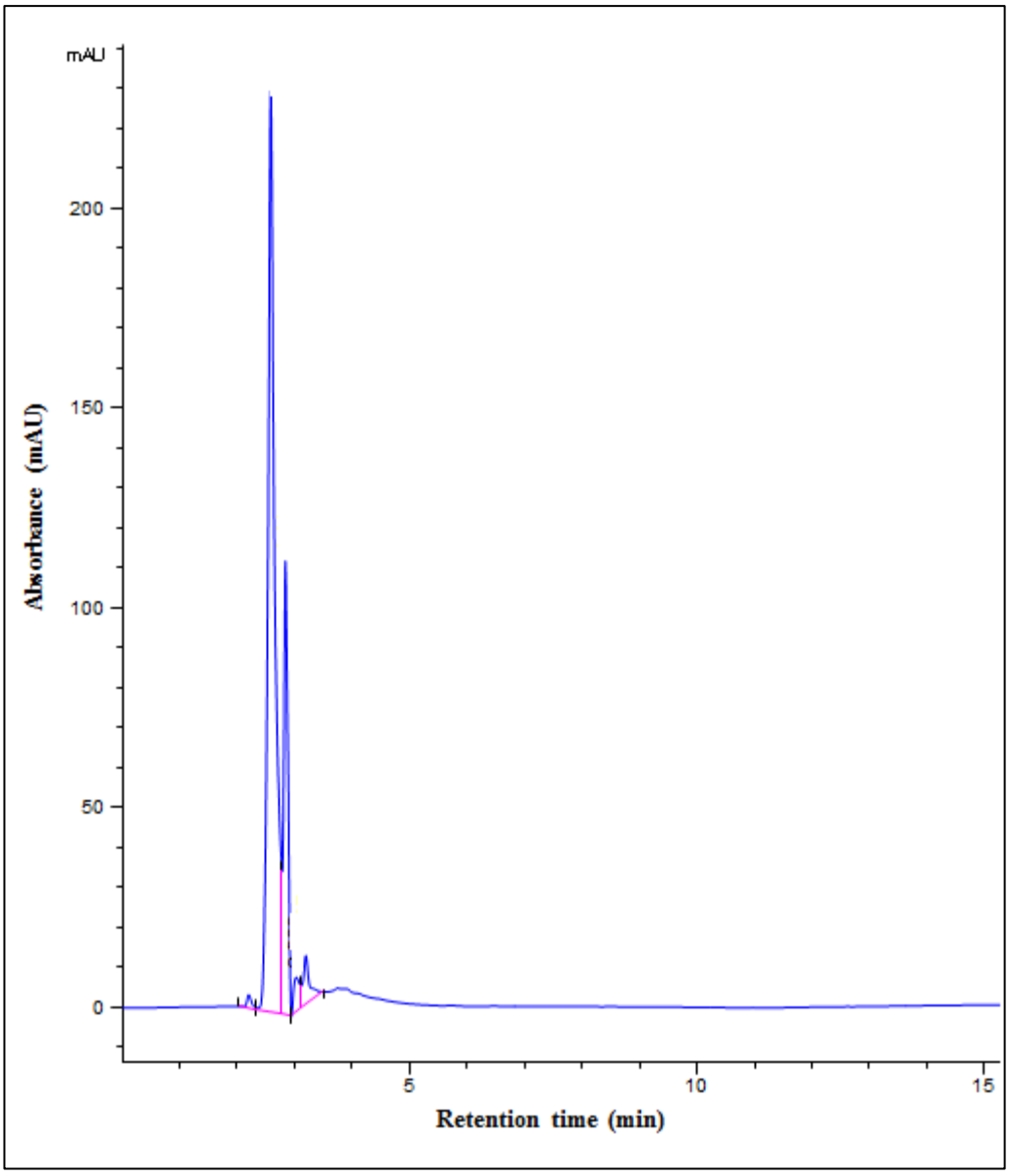

Figure 3.10: Chromatogram obtained after injection of frusemide in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

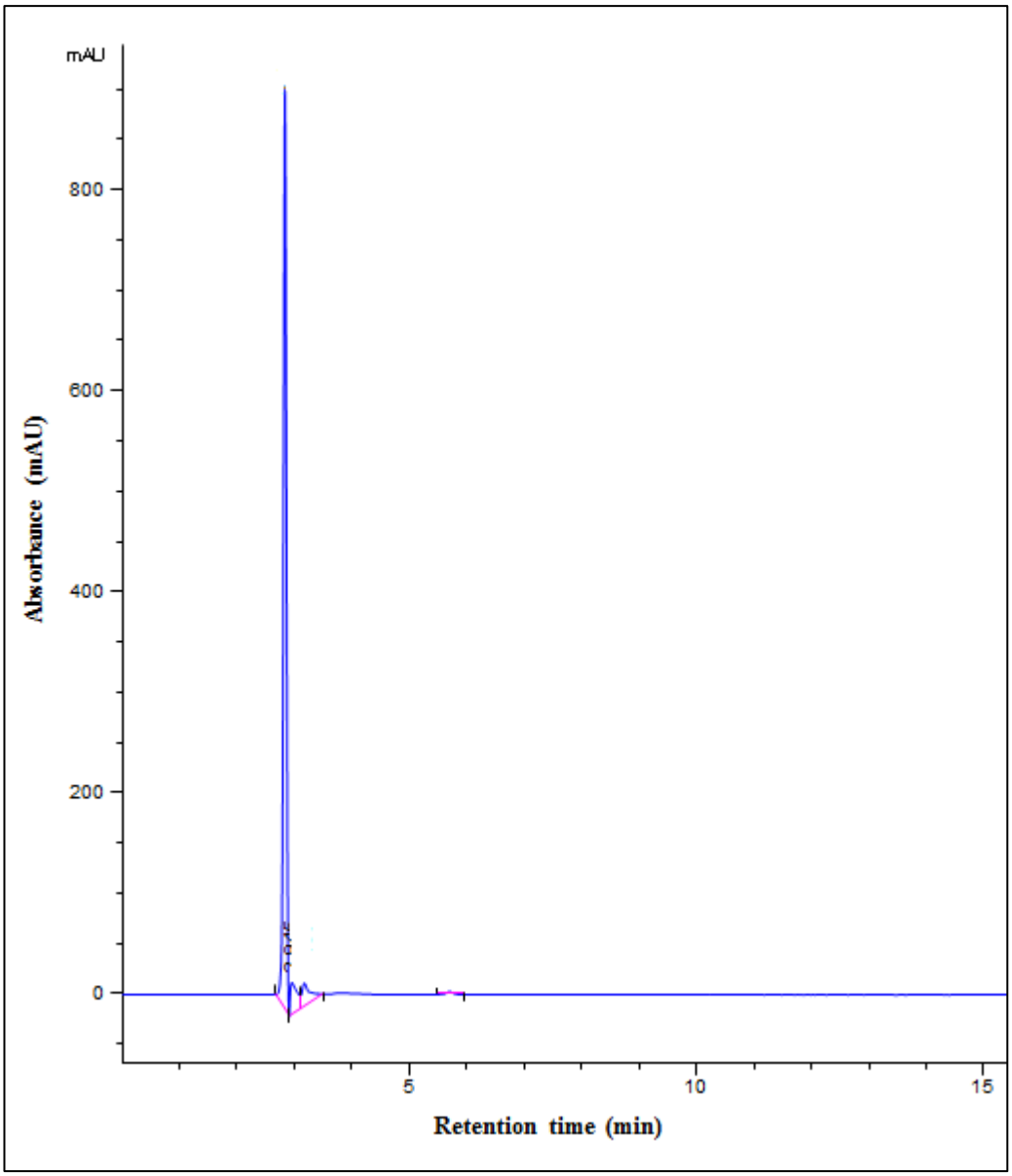

Figure 3.11: Chromatogram obtained after injection of niacinamide in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

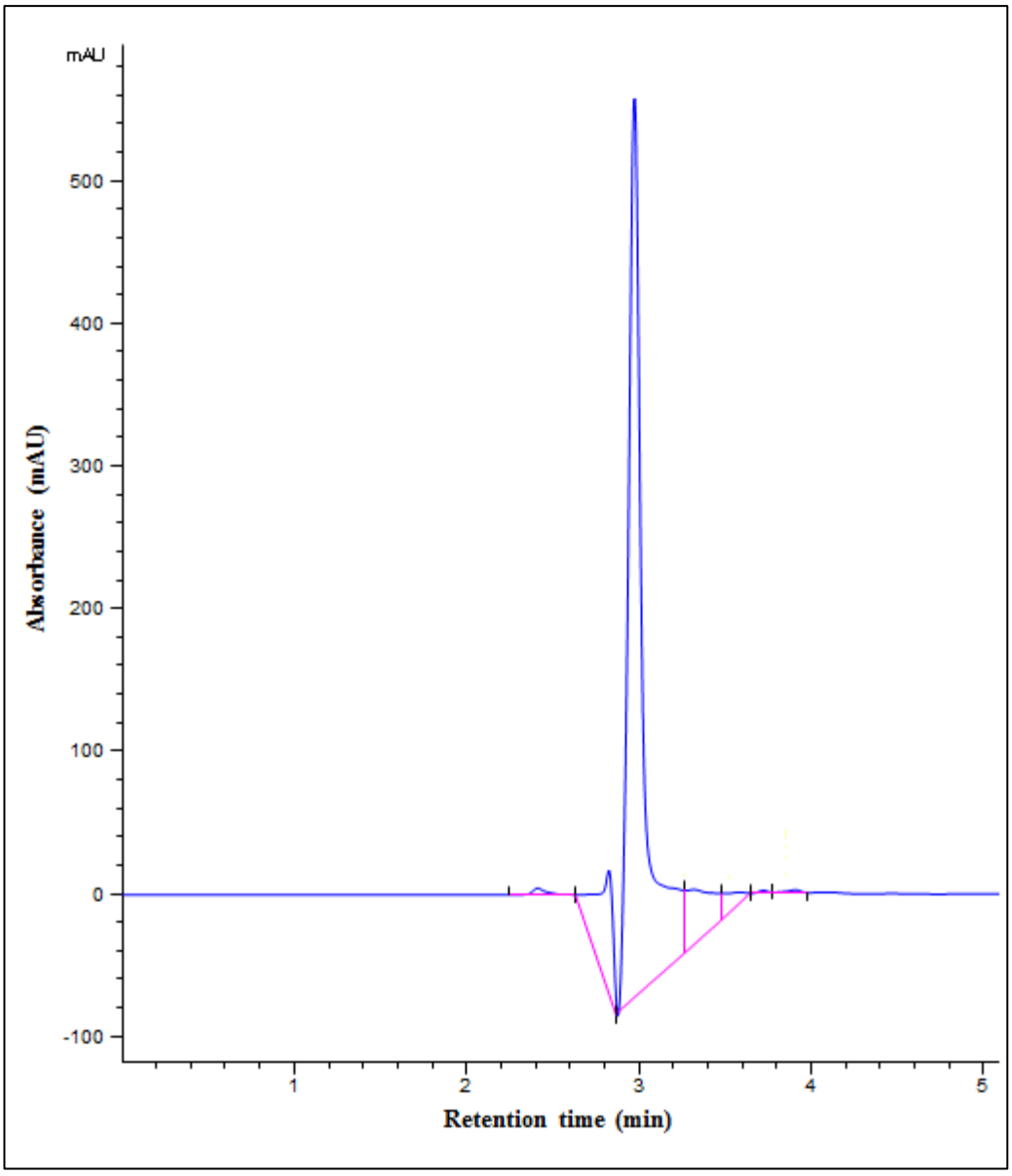

Figure 3.12: Chromatogram obtained after injection of mephenesin in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

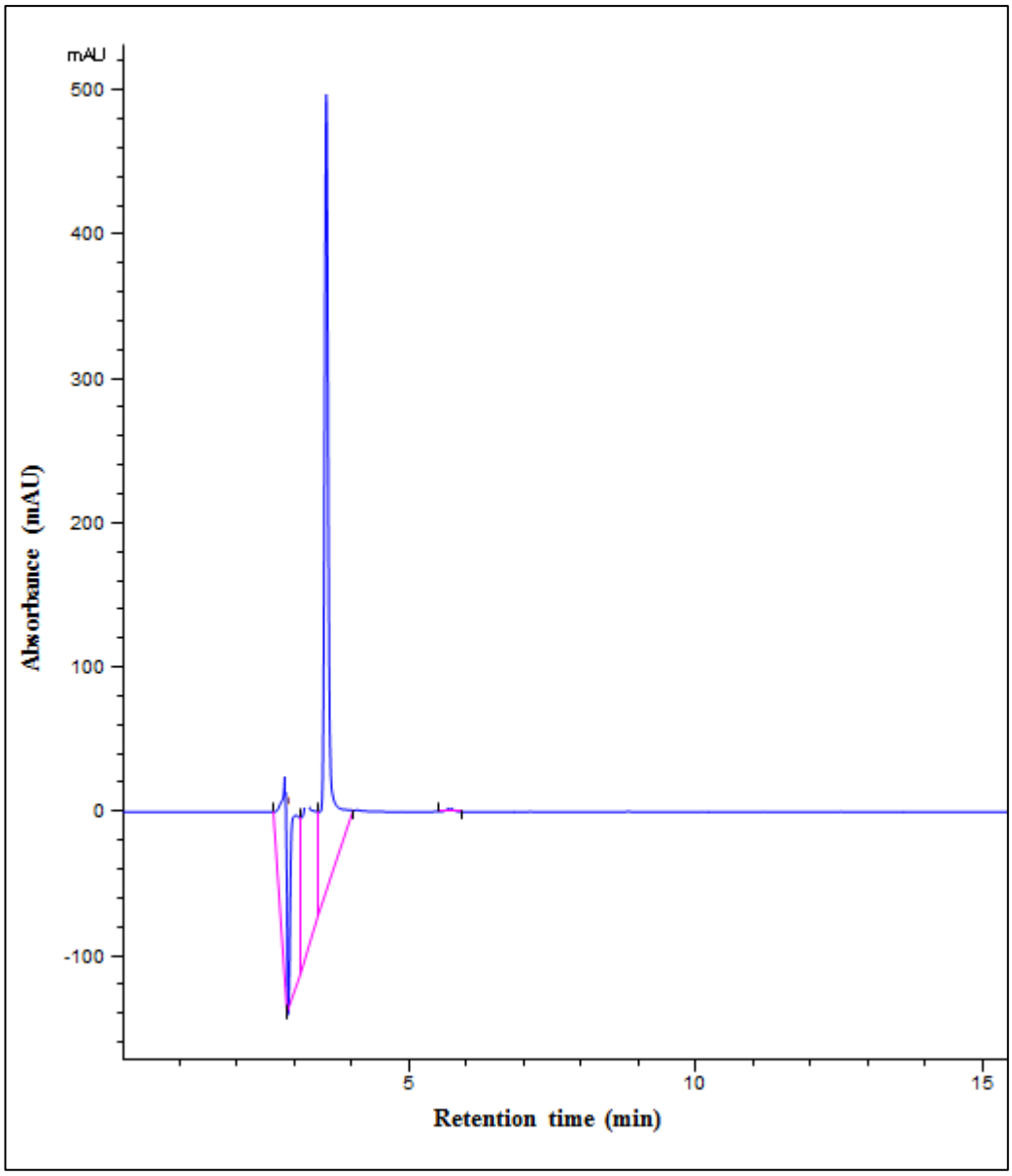

Figure 3.13: Chromatogram obtained after injection of ibuprofen in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

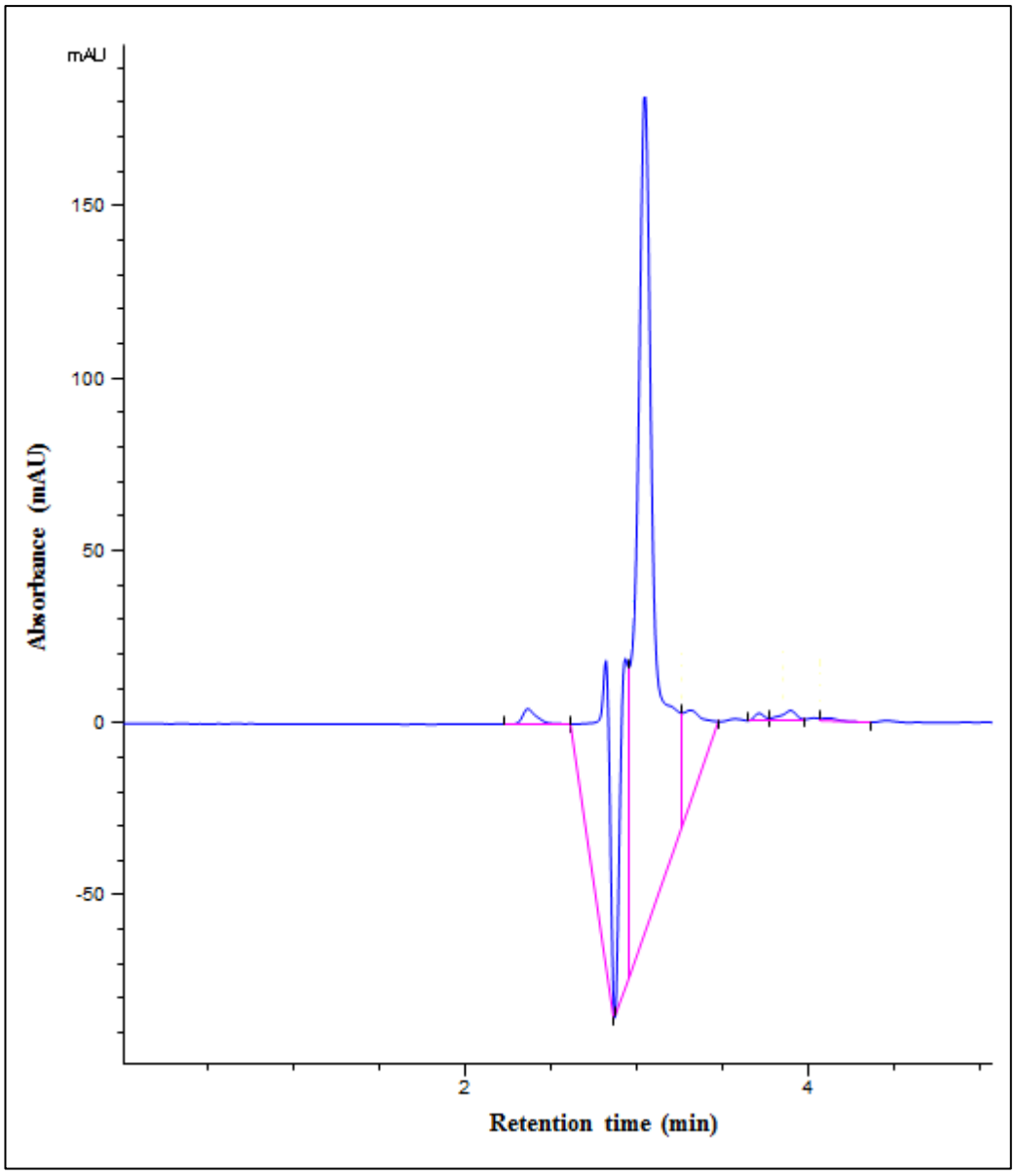

Figure 3.14: Chromatogram obtained after injection of prednisolone in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

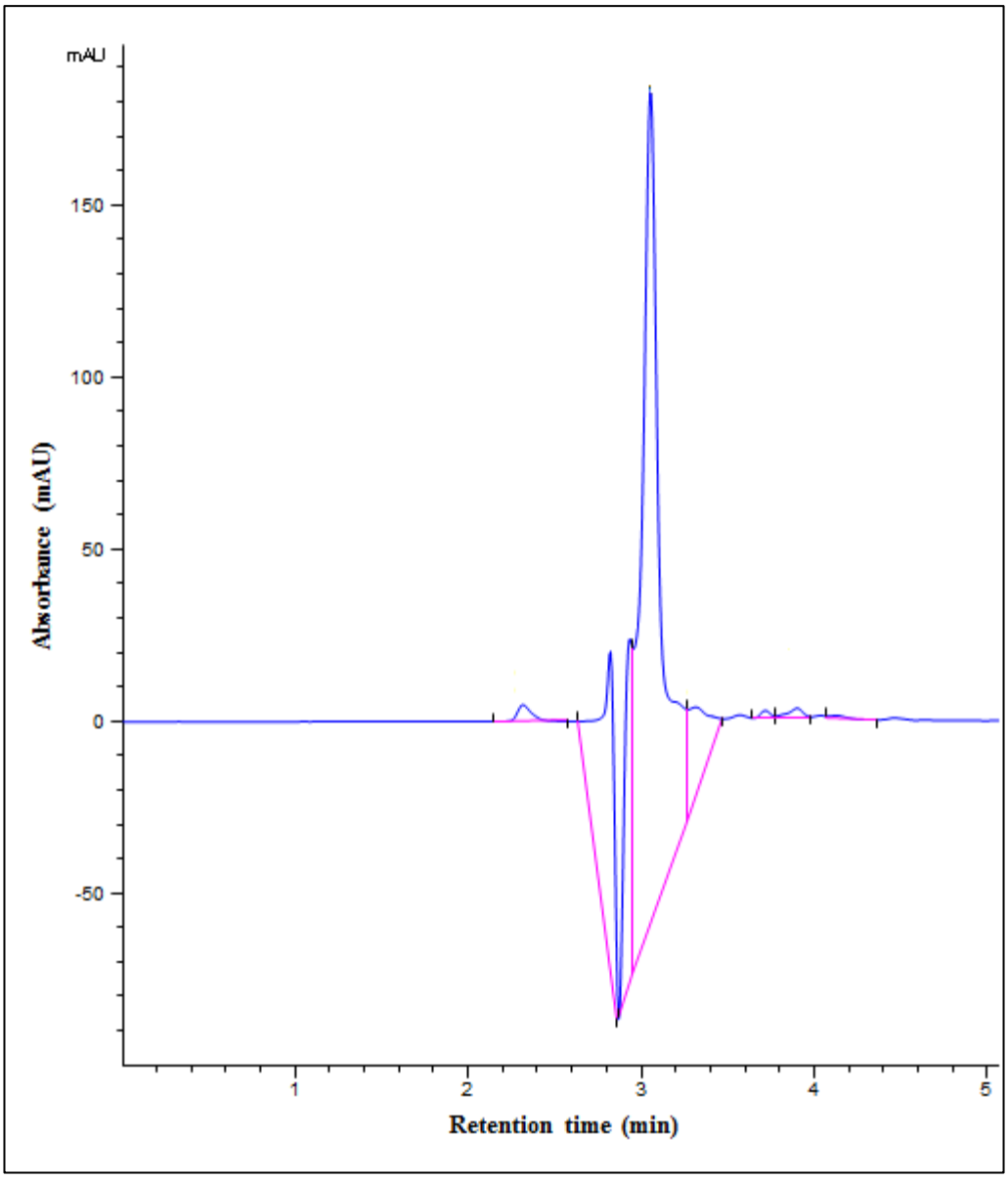

Figure 3.15: Chromatogram obtained after injection of hydrocortisone in methanol (100 µg/ml). No peaks were eluted during the 30 min run time or peaks may co-elute with the solvent front. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

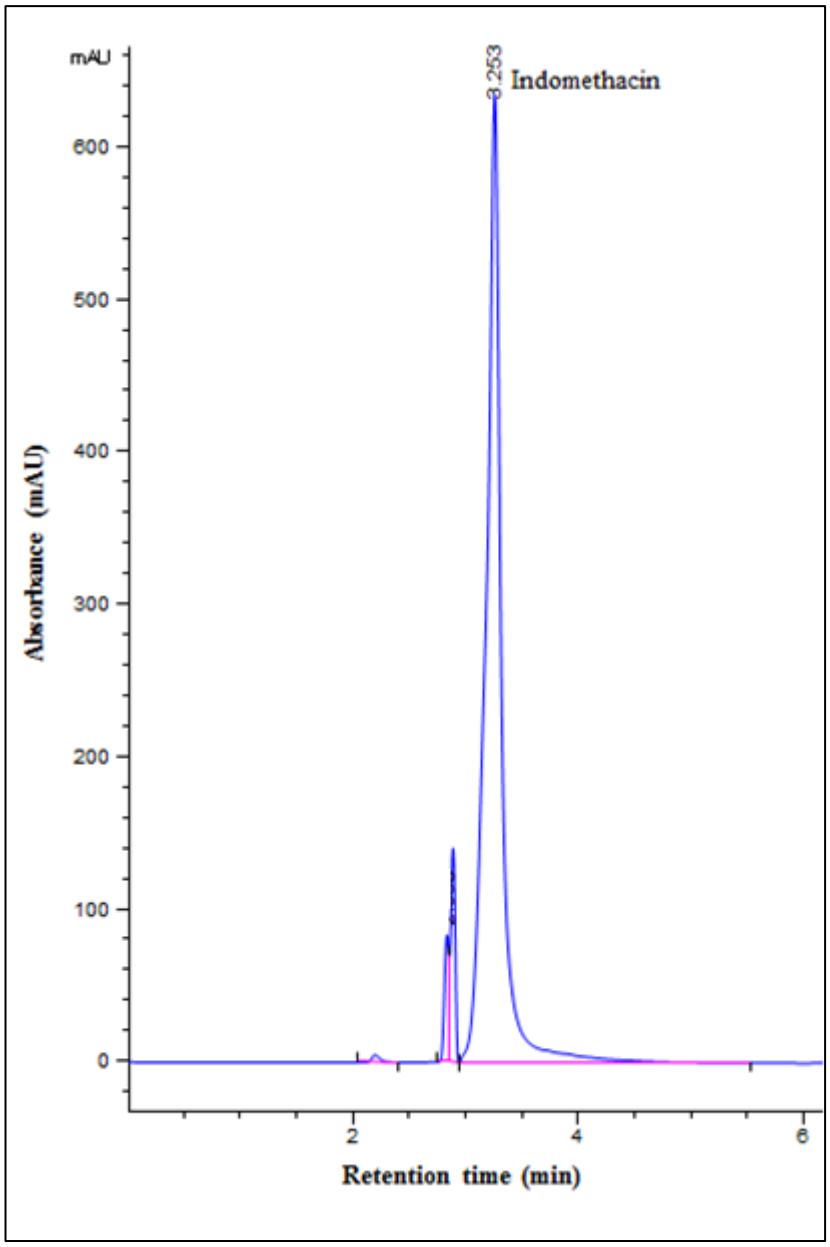

Figure 3.16: Chromatogram obtained after injection of indomethacin in methanol (40 µg/ml). Indomethacin showed a tall and sharp peak at 3.253 min. (**N/B:** Mobile phase composition: 90% acetonitrile, 8% methanol and 2% hexane. Flow rate: 1.0 ml/min. Detection: 208 nm.)

3.1.2 Method development and optimisation

3.1.2(a) Detection

Linoleic acid exhibited two absorption peaks at 208 and 220 nm (Figure 3.17). When linoleic acid was chromatographed at both wavelengths, the area count was bigger at 208 nm. Therefore, 208 nm was selected.

3.1.2(b) Mobile phase

Different combinations of solvents were tested to determine the best mobile phase that would produce the best peak of linoleic acid such as 1) acetonitrile (90%) and water (10%), 2) acetonitrile (90% and methanol (10%), and 3) acetonitrile (90%), methanol (8%) and hexane (2%). From the three combinations, the combination of acetonitrile (90%) and methanol (10%) produce the biggest area under the curve. However the combination of acetonitrile (90%), methanol (8%) and hexane (2%) was chosen because it produced the shortest retention time at 6.38 min and a big peak area. The comparison of peak shapes when using different combinations of mobile phase was shown in Figure 3.18. When acetic acid (0.2%) was added into the selected mobile phase above, a bigger shape of linoleic acid without peak tailing is observed as in Figure 3.19 indicating its suitability.

3.1.3 Validation

3.1.3(a) Linearity

A calibration graph was constructed in the range of 5 to 200 μg/ml linoleic acid concentrations (Table 3.1) for three consecutive days. The linearity of the calibration graph was demonstrated by the good correlation coefficient obtained for the regression line (Figure 3.20).

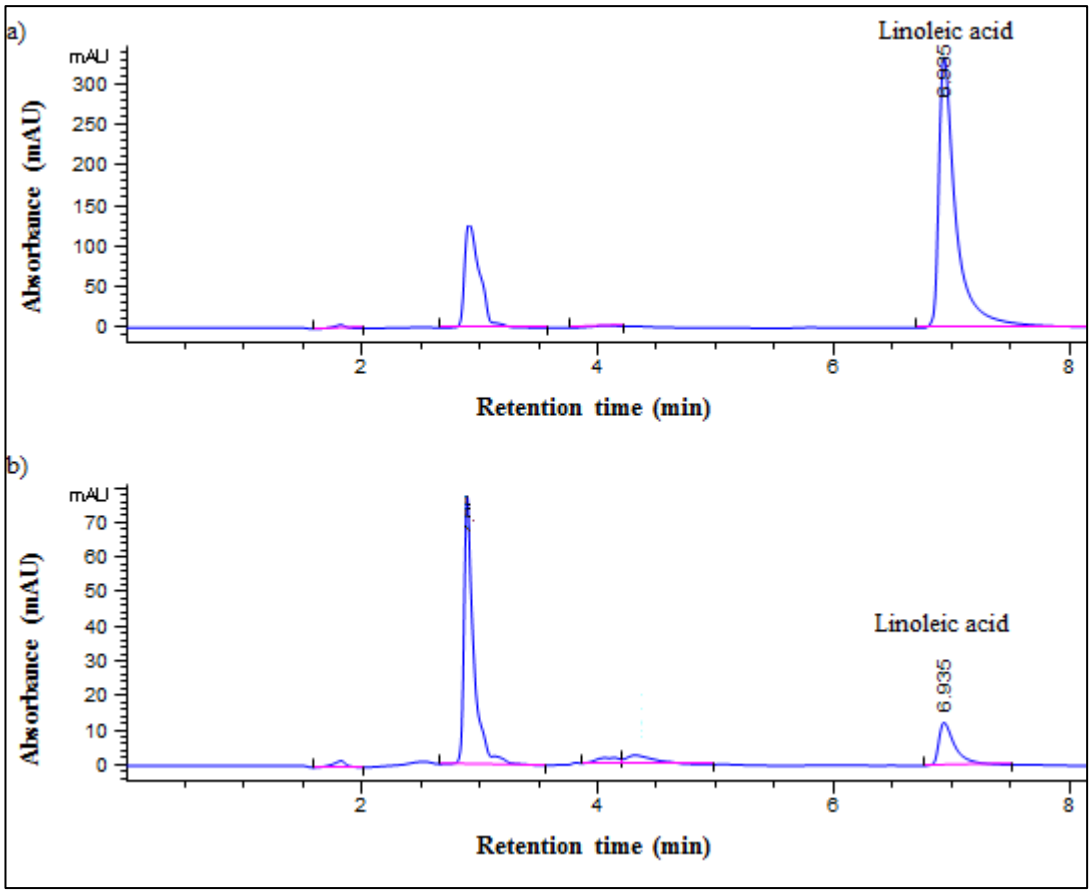

Figure 3.17: Chromatogram of linoleic acid at different wavelengths a) 208 and b) 220 nm. Linoleic acid peak appears bigger and better at 208 nm.

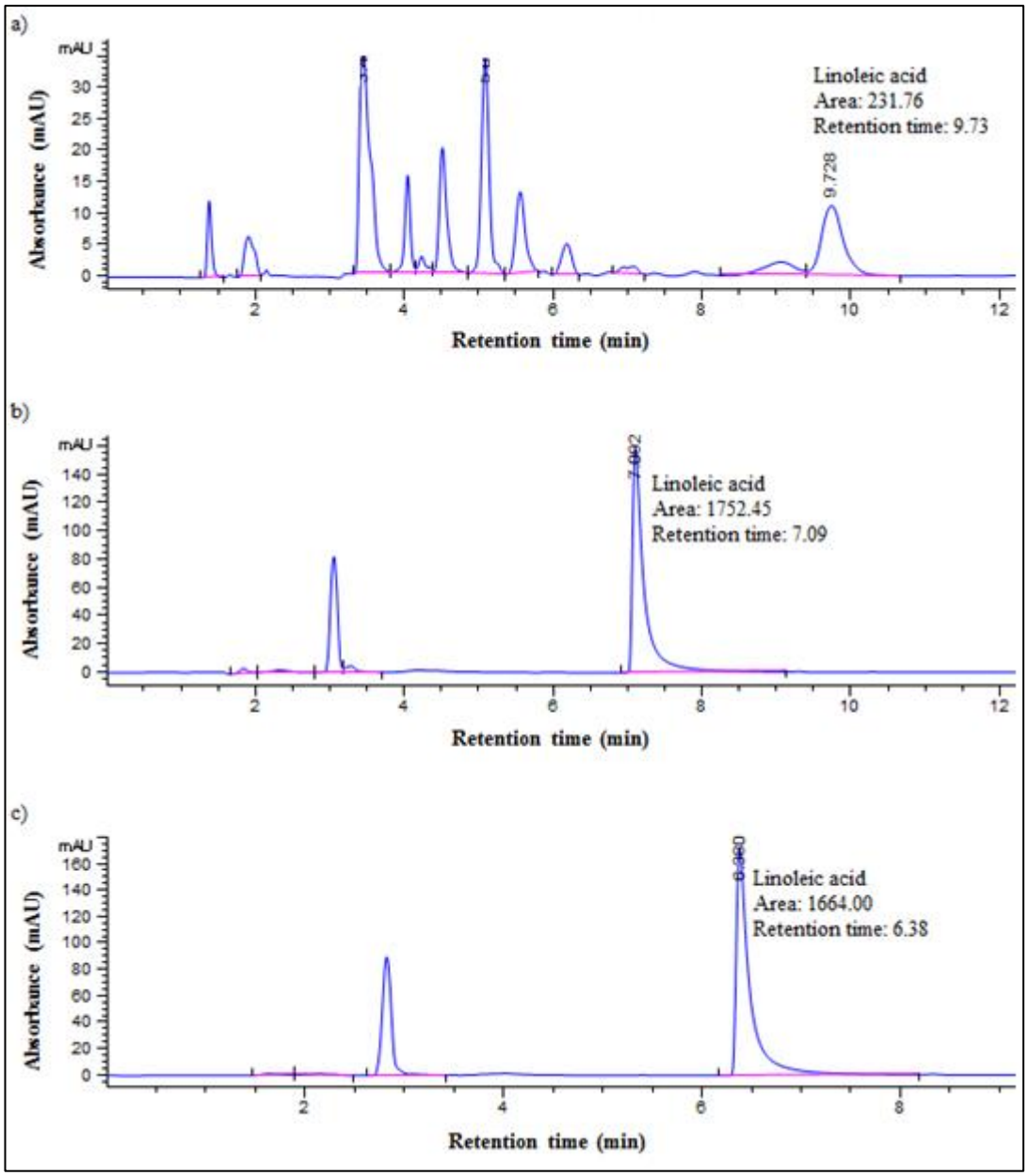

Figure 3.18: Chromatogram of linoleic acid when different combination of solvents were used as mobile phase a) acetonitrile (90%) and water (10%), b) acetonitrile (90% and methanol (10%) and c) acetonitrile (90%), methanol (8%) and hexane (2%). The final combination of acetonitrile, methanol and hexane produce a peak with the shortest retention time.

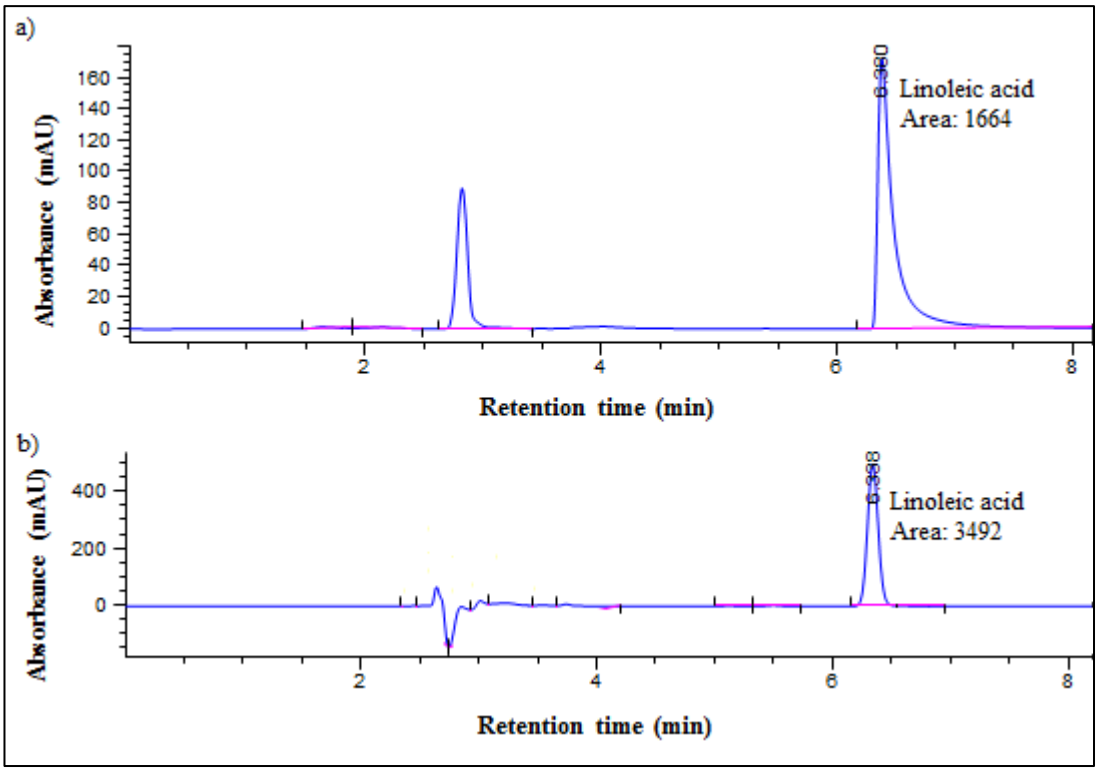

Figure 3.19: Chromatogram of linoleic acid a) without the addition of an organic modifier and b) with the addition of acetic acid (0.2%) as organic modifier. Linoleic acid has a bigger area (without organic modifier: 1664, with organic modifier: 3492) and better peak shape with the addition of an organic modifier indicating its suitability.

Table 3.1 Linoleic acid concentration for calibration graph

Concentration (µg/ml)	Mean area ratio of linoleic acid	Standard deviation	Correlation coefficient
Day 1			
5.000	0.032	0.002	0.9991
15.000	0.087	0.004	
25.000	0.137	0.003	
50.000	0.248	0.018	
100.000	0.536	0.005	
200.000	1.058	0.006	
Day 2			
5.000	0.029	0.005	0.9981
15.000	0.079	0.003	
25.000	0.122	0.006	
50.000	0.262	0.017	
100.000	0.490	0.002	
200.000	1.033	0.027	
Day 3			
5.000	0.033	0.002	0.9981
15.000	0.081	0.006	
25.000	0.131	0.007	
50.000	0.283	0.013	
100.000	0.512	0.005	
200.000	1.099	0.007	

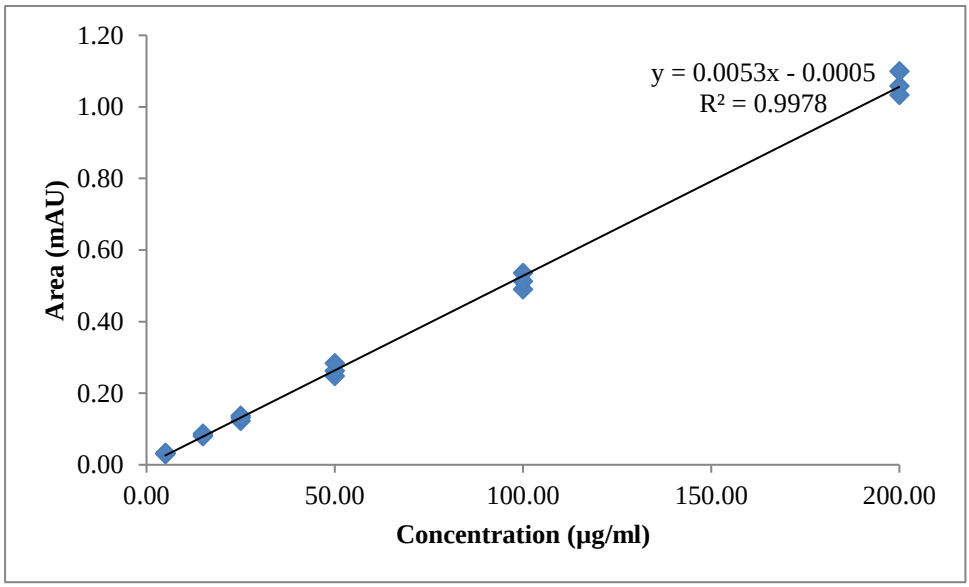

Figure 3.20 Mean calibration graph for linoleic acid.

3.1.3(b) Precision and accuracy

The precision of the test was evaluated by determining the relative standard deviation of the measured peak area ratios for different concentrations of drug. Based on the FDA guideline (2015), the acceptable precision for each concentration is when the relative standard deviation is less than 15%. Accuracy was expressed as the mean percentage of analyte recovered in the assay. The acceptable range of accuracy is within 80-120%. Four concentrations of linoleic acid (80, 90, 110 and 150 μg/ml) were tested in triplicates indicating good precision (Table 3.2). The precision and accuracy tests were performed daily for three days.

3.1.4 Measurement of linoleic acid in *L. rhinocerus* extracts

Table 3.3 shows the amount of linoleic acid in 100 g of the extracts. Based on the results, water-based extracts such as hot water, cold water, hexane residue and petroleum ether residue extracts recorded a low amount of linoleic acid which is approximately 0.05 g in 100 g of extract. Meanwhile, the non-polar extracts such as hexane and petroleum ether yielded significantly higher amounts of linoleic acid which are 16.79 and 17.55 g in 100 g of extract respectively.

3.2 Analysis of β-glucan in *L. rhinocerus* extracts

Analysis of β-glucan composition in water-based *L. rhinocerus* extracts indicated that it is present at 29.0, 33.6, 39.8 and 32.1 g in 100 g extracts of hot water, cold water, hexane residue and petroleum ether residue extracts respectively. On the other hand, the amount of β-glucan in non-polar extracts such as hexane and petroleum ether extracts were 31.5 and 24.1 g respectively. Hexane residue is the best extract for β-glucan (Table 3.4).

Table 3.2 Precision and accuracy of the developed detection method for linoleic acid

Concentration (μg/ml)	Mean	sd	Precision (%)	Accuracy (%)
Day 1				
80.00	81.68	0.46	0.56	102.10
90.00	90.05	3.24	3.60	100.10
110.00	110.13	5.16	4.69	100.11
150.00	148.34	1.83	1.23	98.89
Day 2				
80.00	80.24	1.60	1.99	100.30
90.00	89.23	0.90	1.01	99.14
110.00	108.62	1.89	1.74	98.74
150.00	143.57	0.88	0.62	95.71
Day 3				
80.00	80.31	3.71	4.61	100.38
90.00	88.81	4.23	4.77	98.68
110.00	107.02	5.14	4.80	97.30
150.00	142.32	2.98	2.09	94.88

Table 3.3 Measurement of linoleic acid in *L. rhinocerus* extracts

Extract	Mean concentration of linoleic acid in extract (g) ± standard deviation
Hot water	0.049 ± 0.277
Cold water	0.049 ± 0.115
Hexane	16.790 ± 0.629
Petroleum ether	17.550 ± 0.221
Hexane residue	0.046 ± 0.113
Petroleum ether residue	0.048 ± 0.133

Table 3.4 Analysis of β-glucan in *L. rhinocerus* extracts

Extract	Mean concentration of β-glucan in extract (g) ± standard deviation
Hot water	29.0 ± 0.001
Cold water	33.6 ± 0.006
Hexane	31.5 ± 0.001
Petroleum ether	24.1 ± 0.001
Hexane residue	39.8 ± 0.002
Petroleum ether residue	32.1 ± 0.004

3.3 Cellular studies

3.3.1 Cell proliferation assay

Analysis of cell proliferation by MTS in BEAS-2B treated with various concentrations (250, 125, 62.5, 31.25 and 15.625 µg/ml) of *L. rhinocerus* extracts after 24, 48 and 72 hours of incubation is shown in Figure 3.21. The treatment with hot water and hexane residue extracts has no effect on the proliferation of BEAS-2B cell line at any concentration. Treatment with cold water extract reduces proliferation at 125 and 250 µg/ml on Days 2 and 3. Similar concentration of hexane extract reduces proliferation of BEAS-2B cell line on Day 3. Petroleum ether extract at the concentration of 31.25-250 µg/ml reduces proliferation from Day 1. Among the different extracts, *L. rhinocerus* extracted by hot water recorded the highest cell viability activity [Figure 3.21 (a)]. In contrast, cells treated with 250 µg/ml petroleum ether extract recorded the highest inhibition effect with a viability percentage of less than 20% [Figure 3.21 (d)].

3.3.2 Apoptosis assay

The apoptotic activity of BEAS-2B treated with various concentrations (62.5, 31.25 and 15.625 µg/ml) of various *L. rhinocerus* extracts after 24 hours of incubation is shown in Figure 3.22. Among the extracts, petroleum ether residue recorded the highest apoptotic activity followed by hexane residue and petroleum ether extracts. However, based on statistical analysis, all of the treatment groups are not significantly different from the control group indicating that treatment with *L. rhinocerus* extracts does not induce apoptotic activity in BEAS-2B.

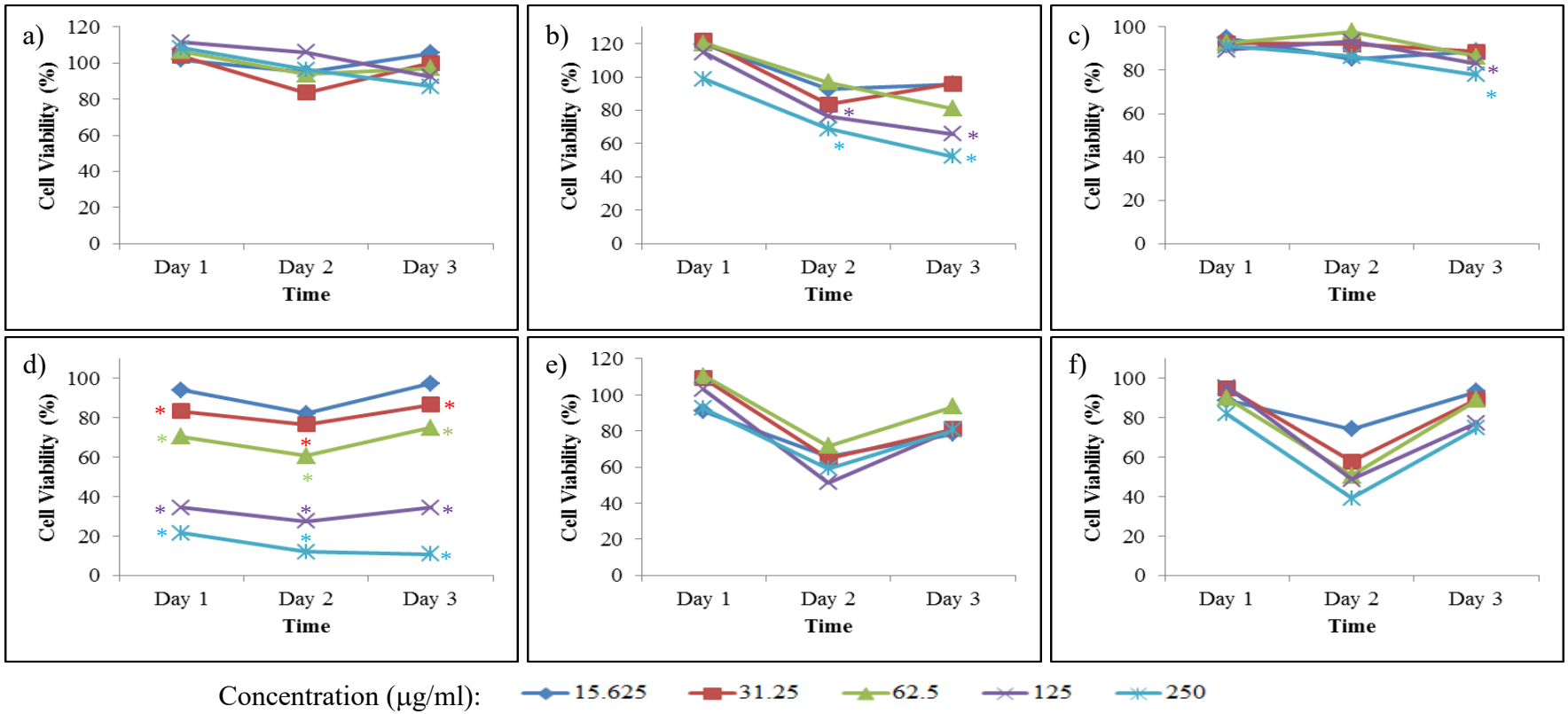

Figure 3.21: The effects of adding various concentrations of *L. rhinocerus* extracts on BEAS-2B cell line for 3 days. Proliferation assay of a) hot water, b) cold water, c) hexane, d) petroleum ether, e) hexane residue and f) petroleum ether residue extracts on BEAS-2B cell line. Untreated cells were used as a control. (* indicates that the treatment is significantly different from control at $p < 0.05$. Different colours represent the different concentrations).

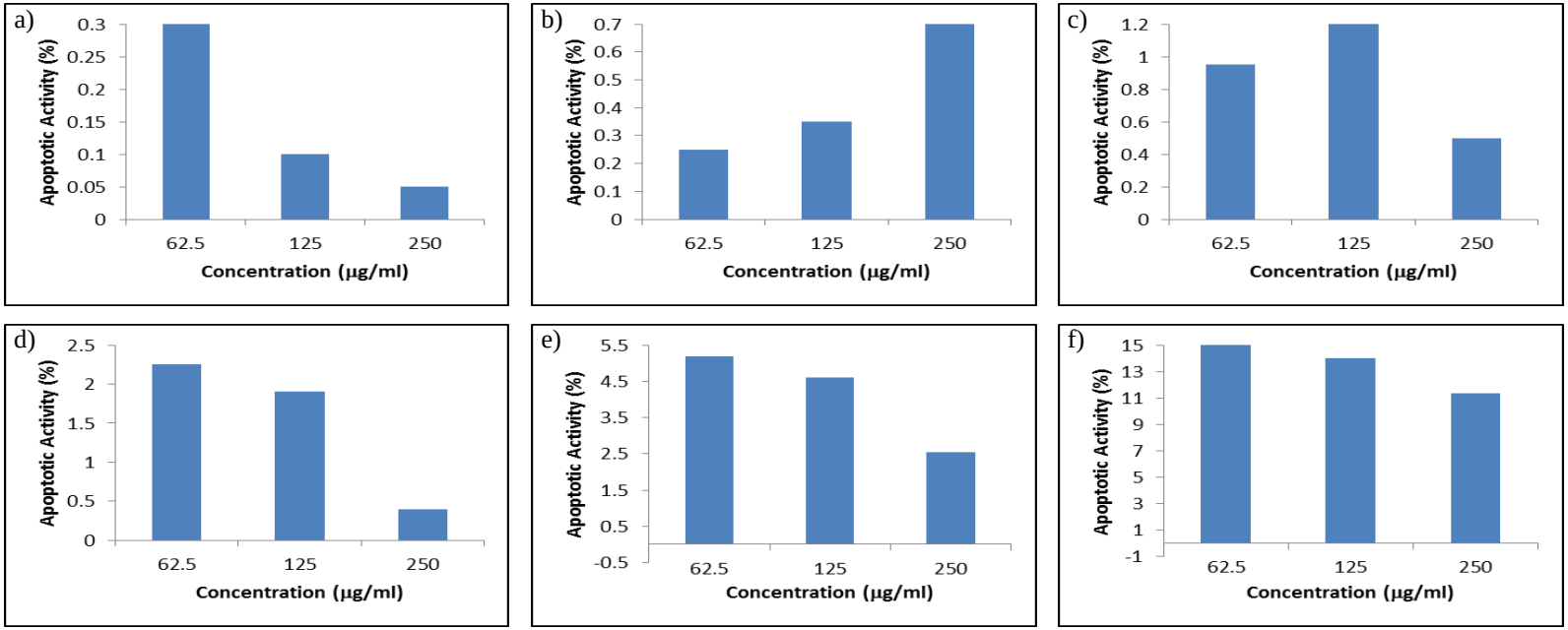

Figure 3.22: The effects of adding various concentrations of a) hot water, b) cold water, c) hexane, d) petroleum ether, e) hexane residue and f) petroleum ether residue extract on the apoptotic activity of BEAS-2B cell line. Untreated cells were used as a control. The differences between the concentrations of each extract were tested statistically using Kruskal-Wallis. The *p* values for a) 0.764, b) 0.493, c) 0.889, d) 0.139, e) 0.208 and f) 0.078 show that none of the extract exhibits significant difference between concentrations indicating that the extracts do not affect apoptosis rate of BEAS-2B.

3.3.3 Cytokine profile analysis

Cytokine profile analysis of BEAS-2B treated with various *L. rhinocerus* extracts at 62.5 μg/ml after 48 hours of incubation is shown in Figures 3.23 – 3.25. All tested groups are not significantly different from the control group indicating that *L. rhinocerus* extracts does not affect the release of T-helper 1/T-helper 2/T-helper 17 cytokines by the BEAS-2B cells.

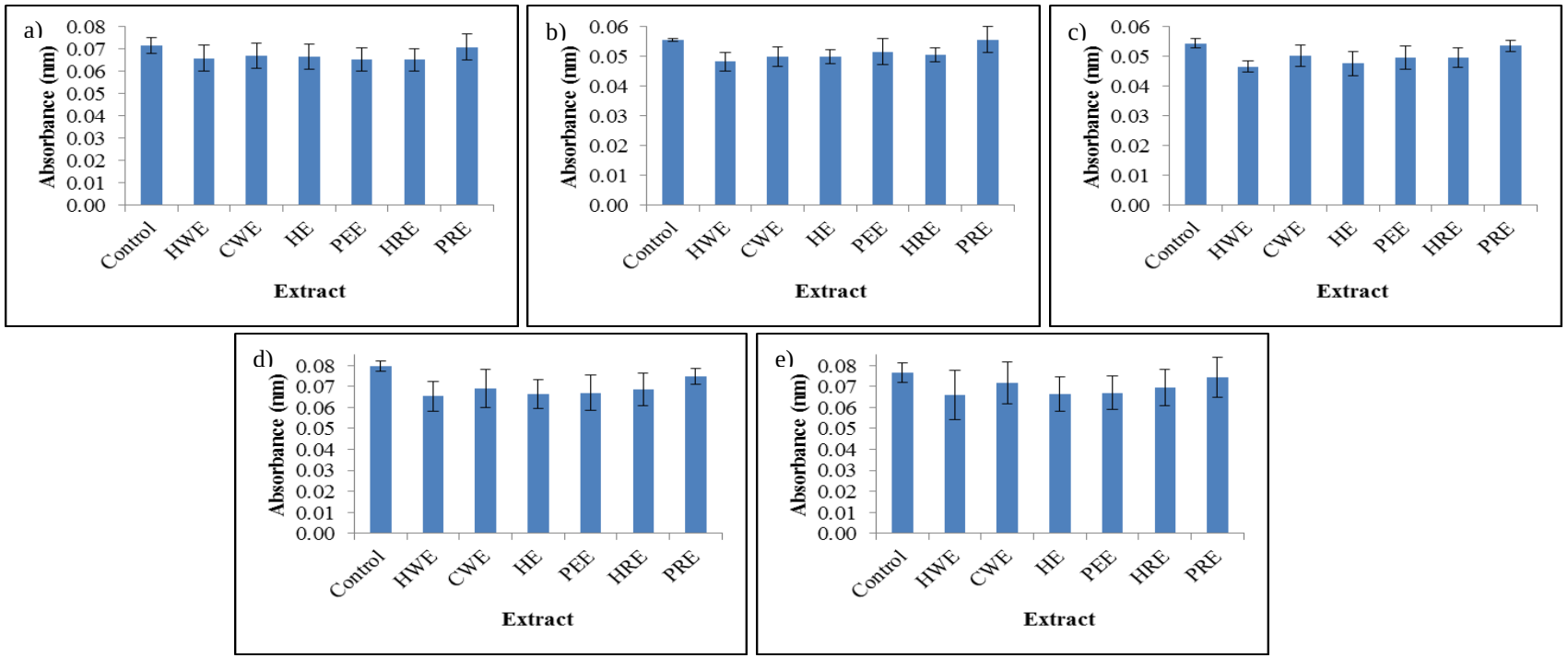

Figure 3.23: T-helper 1 related cytokine profile of BEAS-2B cell line treated with various extracts of *L. rhinocerus*. The effect of various extracts on the release of a) IL-2, b) IL-10, c) IL-12, d) IFN-γ and e) TNF-α cytokines was investigated. Untreated cells were used as a control. The differences between extracts were tested statistically using Kruskal-Wallis. The *p* values for a) 0.843, b) 0.584 c), 0.571 d) 0.474 and e) 0.906 show that none of the extract has significant effect on the release of cytokines by BEAS-2B. (**N/B**: HWE = how water extract, CWE = cold water extract, HE = hexane extract, PEE = petroleum ether extract, HRE = hexane residue extract and PRE = petroleum ether residue extract.)

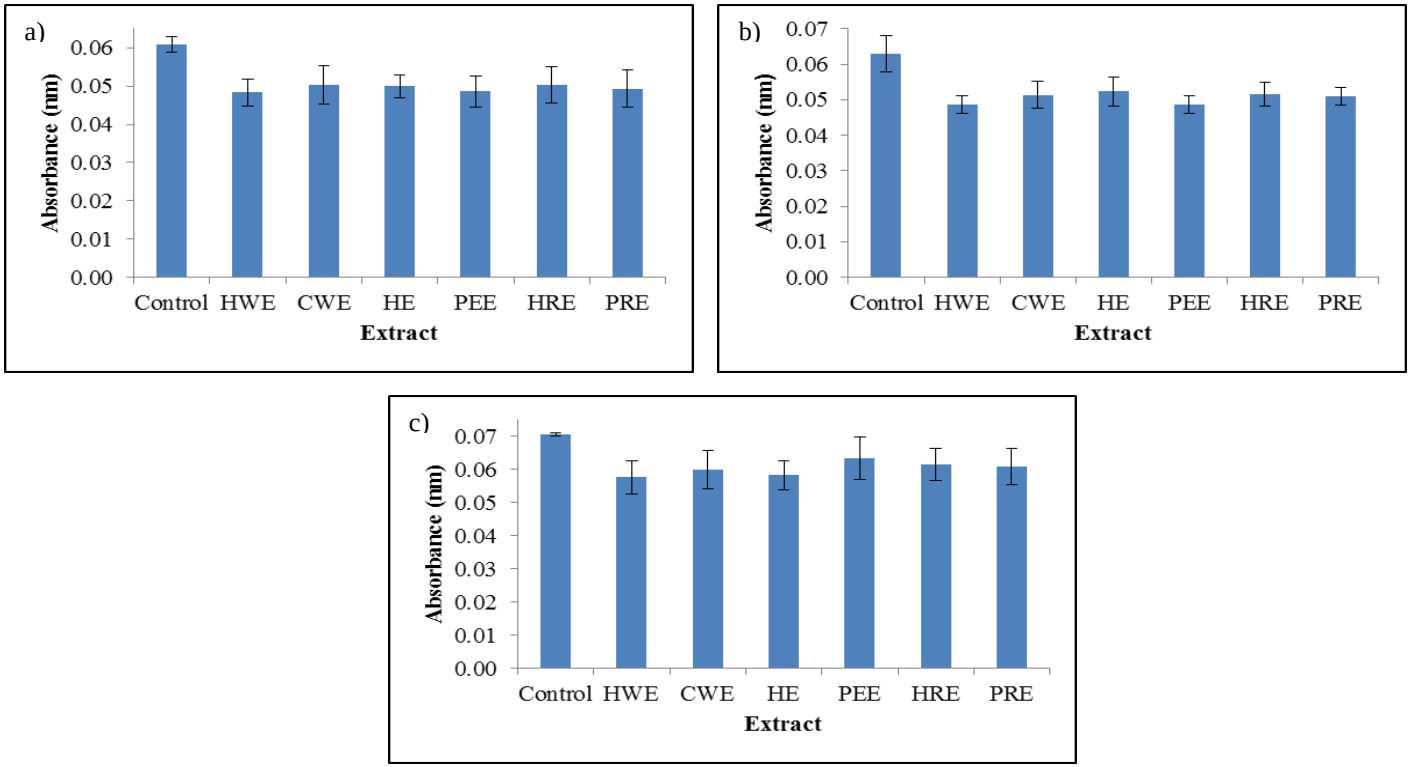

Figure 3.24: T-helper 2 related cytokine profile of BEAS-2B cell line treated with various extracts of *L. rhinocerus*. The effect of various extracts on the release of a) IL-4, b) IL-5 and c) IL-13 cytokines was investigated. Untreated cells were used as a control. The differences between extracts were tested statistically using Kruskal-Wallis. The *p* values for a) 0.591, b) 0.350 and c) 0.484 show that none of the extract has significant effect on the release of cytokines by BEAS-2B. (**N/B**: HWE = how water extract, CWE = cold water extract, HE = hexane extract, PEE = petroleum ether extract, HRE = hexane residue extract and PRE = petroleum ether residue extract.)

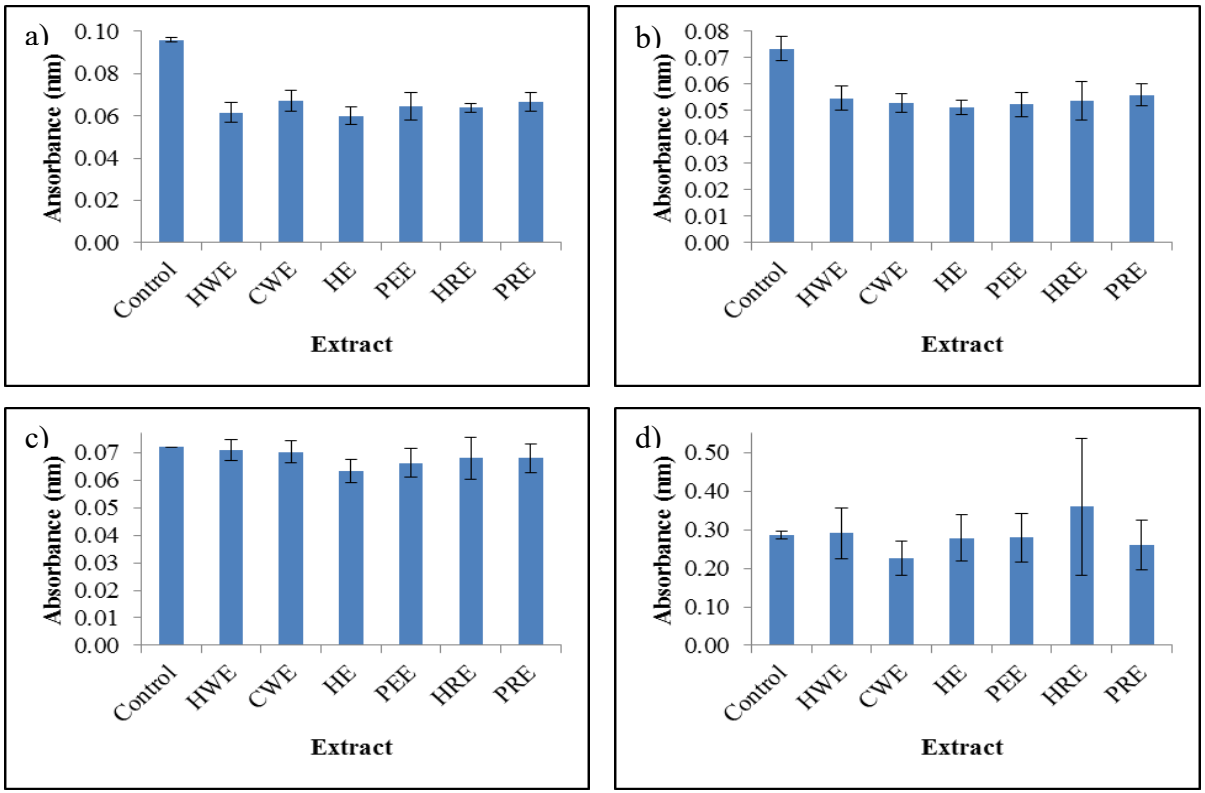

Figure 3.25: T-helper 17 related cytokine profile of BEAS-2B cell line treated with various extracts of *L. rhinocerus*. The effects of various extracts on the release of a) IL-6, b) IL-17A, c) Granulocyte colony-stimulating factor and d) TGF-β1 cytokines was investigated. Untreated cells were used as a control. The differences between extracts were tested statistically using Kruskal-Wallis. The *p* values for a) 0.283, b) 0.389, c) 0.832 and d) 0.955 show that none of the extract has significant effect on the release of cytokines by BEAS-2B. (**N/B**: HWE = how water extract, CWE = cold water extract, HE = hexane extract, PEE = petroleum ether extract, HRE = hexane residue extract and PRE = petroleum ether residue extract.

CHAPTER 4

DISCUSSION

4.1 General discussion

Dietary fat is one of the three main macronutrients important to ensure balanced lipid homeostasis for good membrane protein function and fluidity as well as regulating cell signalling processes, cellular functions and gene expression (Patterson *et al.,* 2012). Although mushroom is generally considered as low fat food with the amount of fat in different species ranging from 1.75-15.50% per 100 g of dried mushrooms, the fat in mushroom is of good quality because it consists of mainly unsaturated fatty acids (Günç Ergönül *et al.*, 2013). Among all of the unsaturated fatty acids, linoleic acid is the dominant fatty acid and is known as the precursor for 1-octen-3-ol or better known as fungi alcohol which is the principal aromatic compound in most fungi that may contribute to the mushrooms' flavour (Woldegiorgis *et al.*, 2015).

Metabolism of unsaturated fatty acid is necessary before some biological processes can occur in the body. For example, linoleic acid has to be converted to arachidonic acid via gamma-linoleic and dihomo-gamma-linolenic acids. Subsequently, cyclooxygenases and lipoxygenases will convert arachidonic acid to prostaglandins, thromboxanes and leukotrienes (Patterson *et al.*, 2012). The lipid mediators will then participate in various pathological processes involving inflammatory conditions including asthma, chronic obstructive pulmonary disease and acute respiratory distress syndrome.

Besides fatty acids, other compounds of interest that may be beneficial to health is β-glucan. The major structural feature of mushroom beta-glucans is a β-1,3-

d-glucan main chain with single d-glucosyl residues linking the β-1,3 subunit to the main chain. Some of the glucans can be extracted from the fruiting body of the mushroom, and soluble β-glucans are also reported to be produced by cultured mycelia (Chang and Wasser, 2012). β-glucan have bioactive properties such as antiviral (Zhang *et al.*, 2007), immunomodulatory, antitumour (Sari *et al.*, 2016), and hepato-protective effects (Wasser, 2014). Since β-glucan is not synthesized by the human body, they are recognized by the immune system and can induce both adaptive and innate immune responses (Brown and Gordon, 2005).

4.2 Detection of linoleic acid in *L. rhinocerus*

Gas chromatography has been widely utilized in fatty acid analysis. For instance, in a study on fatty acid composition of some medicinal mushrooms in Turkey (Türkekul *et al.*, 2017), gas chromatography equipped with a flame ionization detector was used. The detection of fatty acid methyl esters of the mushrooms were achieved using a polar capillary column (30 m × 0.25 mm and 0.25 μm ID). Helium was used as the carrier gas and the column temperature was gradually elevated (from 100^{o}C to 200^{o}C) at 2^{o}C/min. Identification of fatty acid components was accomplished based on the comparison of their retention times with the standards. The peak area percentages of compounds were calculated based on the flame ionization detector data.

Although gas-chromatography is a reliable method, it is only suitable for volatile compounds. Linoleic acid is difficult to be directly separated by gas chromatography because of its low volatility (Qu *et al.*, 2015). Hence, to employ this method, the non-volatile samples such as *L. rhinocerus* extracts need to be derivatized first (Tarola *et al.*, 2012). In case of detecting fatty acid, the samples have to undergo thermal and chemical degradations to produce fatty acid methyl ester.

(Tarola *et al.*, 2012). However, sample preparation for analysis of linoleic acid in extracts is complicated and time consuming. These procedures have to face the problems associated to an incomplete derivatization and the residual hydrolysis product when used in real samples (Zhou *et al*., 2013). In contrast, the method optimized in our study can readily detect linoleic acid without the need of derivatization of samples. Other drawbacks of gas chromatography are low stability of compounds, harmful derivatization reagents and interferences (Zeng *et al.*, 2017). In comparison to the gas chromatography, analysis of fatty acids by HPLC is performed under mild and friendly conditions, thus avoiding the disadvantages mentioned. Therefore, HPLC is a popular alternative to gas chromatography.

One of the important steps that should be performed prior to phytochemical analysis is extraction of sample materials. This step is important because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Hot and cold water extraction was employed in our study because there are many studies reported on the potential of hot and cold water extracts of *L. rhinocerus.* For instance, hot water extract of *L. rhinocerus* is reported to stimulate neuritogenic activity in PC-12 cells (Seow *et al.*, 2015). Hot water and cold water extracts of *L. rhinocerus* are also reported to have anti-inflammatory (Lee *et al.*, 2014) and anti-oxidative (Yap *et al.*, 2013) potentials. Therefore, we would like to elucidate the potential of hot and cold water *L. rhinocerus* extracts on pulmonary cells. Besides, water is an interesting choice of solvent because several of its chemical and physical properties largely change according to the temperature (Brunner, 2014). A higher temperature results in faster diffusivity as well as altered solubility. Therefore, the higher the temperature, the more unwanted compounds are extracted leading to lower selectivity (Plaza and

Turner, 2015). When water is used as a solvent for extraction, it has almost an insignificant impact on the environment.

There are several methods to extract *L. rhinocerus* available in the literatures. A study (Lau *et al.*, 2013) reported on analysis using hot and cold water extracts of *L. rhinocerus*. The chemical composition and cellular toxicity of *L. rhinocerus* in hot water extract were analysed after boiling powdered sclerotium in distilled water (1:20) at 90–95°C for 60 min, while the cold water extract was prepared by subjecting the mixture at similar ratio to continuous stirring at 4°C for 24 h. The preparation of cold water extract in our study is similar to the method employed by Lau *et al*. (2013). However, soxhlet extraction method was used in our study to prepare hot water extract because as compared to boiling, in soxhlet extraction the sample is repeatedly brought into contact with fresh portions of extractant, hence the extraction process is more effective. Also, no filtration is required after leaching because *L. rhinocerus* powder was placed in a thimble.

Moreover, soxhlet extraction is a very simple, well-established methodology that can extract more sample mass than most of the latest alternatives (De Castro and Priego-Capote, 2010). The extraction and evaporation temperatures have a significant effect on the quality of final products. Mamidipally and Liu (2004) found that the oil extracted rice bran using soxhlet method was slightly darker compared to hexane extracted oil, probably due to higher extraction and evaporation temperatures used during the extraction process. A similar pattern is observed in hot water extract in our study. Hot water extract appeared to be darker as compared to the other extracts which may indicate higher antioxidant contents.

Our study also focuses on preparing extract that has high fatty acid content. Many studies have reported on the use of ultrasound-assisted extraction for fatty acid extraction. For example, raspberry oil extracts in oil which was subjected to an ultrasound-assisted extraction exhibited a higher fatty acid content as compared to oil subjected to soxhlet extraction (Teng *et al.*, 2016). Another example is as reported in a study on rice lipid production (Xu *et al.*, 2016). The study reported no significant differences in the fatty acid composition were observed between the two methods (ultra-sound assisted extraction and soxhlet extraction) suggesting ultrasound has no impact on the rice lipid constituent. Similar results were reported in the investigation of the effect of ultrasound-extraction on the fatty acid compositions of other oilseeds including chickpea (Lou *et al.*, 2010) and flaxseed (Zhang *et al.*, 2008) indicating that the ideal extraction method is very much dependant on the composition of the materials to be extracted.

Although ultrasound-assisted extraction is similar to soxhlet extraction, it is gaining popularity since it is considered as a cleaner method that does not require a lot of solvent or the use of capacious vessels like soxhlet. It is also better in terms of energy and time. This is as confirmed by a study on the extraction of oleaginous seeds that indicated soxhlet extraction procedure to be not only time consuming but also requiring higher energy (8 kW.h) as compared to only 0.25 kW.h for ultrasound-assisted extraction (Chemat *et al.*, 2017). The use of ultrasound intensifies the mass transfer and accelerates the access of solvent into cell of sample materials. With the help of ultrasound, the diffusion of solvent across the cell wall and the rinsing of contents of cell after breaking the walls is faster and more effective (Azmir *et al.*, 2013). Hence, ultrasound-assisted extraction was employed to obtain extract with high fatty acid content.

The solvent choice in ultrasound-assisted extraction depends on the solubility of the target metabolites (Chemat *et al.*, 2017). According to the "like-dissolve-like" rule, non-polar compounds dissolve in non-polar solvents. In case of our study, the target metabolite is linoleic acid, which is a largely non-polar fatty acid that dissolves in non-polar solvents such as hexane and petroleum ether. Many studies advocate the use of hexane as a solvent for fatty acid extraction. For example, in the analysis of lipid and fatty acid profile of *Laetiporus sulphurous* an edible fungus, hexane was used as the solvent in the extraction method due to the high presence of its non-polar constituents (Sinanoglou *et al.*, 2015). Fatty acid analysis of mango kernel oil revealed that the oil extracted with hexane has the best yield of linoleic acid as compared to ethanol and petroleum ether (Kittiphoom and Sutasinee, 2013). Some also reported the use of petroleum as solvent to extract fatty acid including the study of fatty acid compositions of *Trifolium angustifolium* (Ertaş *et al.*, 2015) and the analysis of fatty acid in *Alcea pallida* and *Alcea apterocarpa* (Ertas *et al.*, 2016) based on the similar principle of non-polar solvents attracting non-polar compounds.

The extraction of β-glucan is more tedious since special attention is needed in order to produce a good and consistent raw material (Ahmad *et al.*, 2012). This is due to the fact that the nature of the extraction method will affect the molecular weight of β-glucan produced, which at the same time will greatly affect their functional behaviours (Brennan and Cleary, 2005). The extraction procedure depends on the solubility of β-glucan in hot water and in alkaline solutions (Zhu *et al.*, 2015). One of the methods to extract β-glucan from *Agaricus blazei* involved a maceration technique in distilled water (100°C) for 3 hours (Kim *et al.*, 2005). Another study on the isolation of β-glucan from *L. rhinocerus* revealed two methods of isolating polysaccharide glucan using two different solvents which are

water and alkaline solution (Mohd Jamil *et al.*, 2013). Water-based β-glucan was prepared by extracting the dried powder of the fruit body twice with distilled water at 100°C and for one hour. On the other hand, the alkaline-based β-glucan was prepared by extracting the dried fruit body powder twice with 1.25 M sodium hydroxide at 60°C for one hour. After one hour, the alkaline extract was neutralized using hydrochloric acid. Both water and alkaline extracts were subjected to centrifugation before the supernatants from both extracts were collected and resuspended in distilled water while being subjected to an overnight shaking condition to remove low molecular weight substances. Two volumes of absolute ethanol were added to both extracts and were left overnight at 4°C for complete precipitation. The solid fractions known as polysaccharide glucan were collected by centrifugation and oven-dried at 60°C. The β-glucan recovered was 0.956% w/w for the hot water extract and 14.433% for alkaline extract. Although we did not employ any extraction method focusing on the extraction of β-glucan, however the recovery of β-glucan in our study is higher which is in the range of 25-40% w/w for all extracts. This is probably due to the extraction method employed in our study also used high temperature (soxhlet extraction) at longer period. In case of the other extracts, the use of agitation in cold water extract and ultrasound in hexane and petroleum ether extracts might facilitate the extraction of β-glucan.

One of the important components in HPLC analysis is the internal standard. The use of internal standard is critical in maintaining the accuracy and precision of the method. With the use of an internal standard, the errors that may occur from many variables during sample preparation and the variations introduced to sample volume can be corrected (Liyanaarachchi *et al.*, 2017). In addition, variations arising from fluctuations in flow rates, temperatures, detector response occurring between

runs which affect the response of the analyte of interest can also be compensated with the use of internal standard (Liyanaarachchi *et al.*, 2017).

Several factors should be considered in the search for a suitable internal standard, 1) the internal standard should be completely resolved from all peaks in the sample and 2) it should be eluted near the analyte and not normally present in the sample (Kuwana, 2012). From the results, only indomethacin is suitable to be used as internal standard because it fulfils all the requirements while others such as 4-hydroxy-3-methoxyphenylacetic, benzoic, caffeic, carminic, chlorogenic, L-ascorbic, p-coumaric, trans-cinnamic acid, frusemide, niacinamide, memphenesin, ibuprofen, prednisolone and hydrocortisone may have high polarity causing them to be co-eluted with the solvent fronts.

While HPLC is used to separate linoleic acid from other compounds, detection of linoleic acid was performed with the help of a built in ultraviolet diode-array detector in the HPLC system. Although most fatty acids lack in suitable chromophores, the detection of fatty acids using an ultraviolet detector is deemed suitable due to the presence of a double bond which results in fatty acids molar absorptivity sufficient to achieve a signal peak which is adequate for detection between 200 and 250 nm (de Oliveira *et al.*, 2014). In this study, linoleic acid exhibited a better peak shape at 208 nm as compared to the other investigated wavelength at 220 nm. This finding is similar to that of another study on quantification of underivatized fatty acids from vegetable oils (Guarrasi *et al.*, 2010). However, as organic solvent also absorb at lower wavelength, noise peaks are expected to appear during analysis.

The final selected mobile phase was a combination of acetonitrile, methanol and hexane (90:8:2) with the addition of 0.2% acetic acid which is suitable for a low polarity compound such as linoleic acid. Due to the weak polarity of linoleic acid, non-aqueous solvent system was chosen to improve the separation. This is in accordance with several previous studies. For instance, Liu *et al.* (2013) had accomplished the separation of cycloartenyl ferulate and 24-methylene cycloartanyl ferulate after testing a series of low-polar solvent systems consisting of methanol, acetonitrile and isopropanol. A mixture of hexane and acetonitrile (1:1) turned out to be the best combination for purification in the study. Non-aqueous solvent systems were also employed in the purification of low polar compound, shionone. In the study, several hydrophobic solvent systems were tested. Combination of *n*-hexane and methanol (2:1) as well as heptane, dichloromethane and acetonitrile (20:7:13) were suitable for the separation (Wang *et al.*, 2012).

The carboxylic group in linoleic acid often bind strongly to column active sites, which can impede detection limits and lead to poor peak shapes (Ford *et al.*, 2007; Hallmann *et al.*, 2008). To avoid this, derivation of fatty acid to their non-polar derivatives were done to minimise the unwanted interactions (Ford *et al.*, 2007; Hallmann *et al.*, 2008). However, the procedure can be laborious, time-consuming and a frequent source of error (Darko and Thurbide, 2017). The addition of mobile phase additives such as acetic acid is highly recommended to improve separation and detection of lipids (Cajka and Fiehn, 2014). In this study, the peak shape was improved after the addition of 0.2% acetic acid. A similar trend is reported in a study on separation and quantification of carotenoids in vegetable crops. In the study, the addition of 0.1% acetic acid to the mobile phases was found to improve peak shape

by minimising peak broadening for all compounds (Maurer *et al.*, 2014) as also seen in our study.

Validation of a method is the process by which a method is tested by the user for accuracy and precision of the method. The first step in this method validation was to construct a calibration curve. The method is considered as linear because there is a directly proportional relationship between concentration of the analyte and the response produced with correlation coefficient of 0.9953. The linearity of linoleic acid standard was observed at 5-200 μg/ml which is within the required investigation range that complies with the FDA guideline (Food and Administration, 2015).

Precision of an analytical procedure is defined as the closeness of the data between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed condition. There are three levels that need to be considered in order to determine the precision of a method such as repeatability, intermediate precision and reproducibility. Repeatability represents the precision of data obtained under similar operating conditions over a short interval of time. Intermediate precision represent the data obtained under certain variations within laboratories, such as the experiment conducted on different days or by different operators or using different equipment. Reproducibility represents the precision between laboratories. The data obtained from our study showed that this method is repeatable and has good precision since the coefficient of variation for quality controls obtained in a single assay as well as on different days was within the acceptable range (not exceeding 15% of the coefficient of variation for all the analytes and less than 20% of the coefficient of variation for the lower limit of quantification as stated in the published guideline) (Food and Administration, 2015).

However, the reproducibility of this method is not proven because the variabilities between laboratories was not performed.

Accuracy is the closeness of the experimental value to the true value of the substance in a sample. There are multiple ways to assess accuracy of a method. First, accuracy can be assessed by comparing the data obtained with an established reference method. Secondly, assessment of accuracy can be done by analysing a sample with known concentrations and comparing the measured value with that of the true value of a reference material supplied. However, if certified reference materials or control samples are not available, a blank sample matrix of interest can be spiked with a known concentration. The latter method was employed in this study and the results show that the method is accurate since the value obtained falls within the acceptable range (not exceeding 15% of the coefficient of variation for all the analytes and less than 20% of the coefficient of variation for the lower limit of quantification as stated in the published guideline) (Food and Administration, 2015).

There are other studies that employ HPLC in the detection of linoleic acid. Tarola *et al.* (2012) reported the detection of fatty acids in drying oils like linseed, walnut and poppy seed that are used in paintings. The separation was achieved on reversed-phase C_{18} column (15 cm x 4.6 mm, ID 3 µm particle size) and the detection was monitored by an ultraviolet detector at 242 nm. The mobile phase used was the combination of acetonitrile and water in the ratio of 85:15 at a flow rate of 1.0 ml/min. This method is cost effective because only one organic solvent which is acetonitrile (instead of three that were used in our study: acetonitrile, methanol and hexane) were used and the column is shorter hence there were reduction in the quantity of mobile phase used. However, the retention time for linoleic acid is longer

and this procedure requires an additional step which is the derivatisation of fatty acid thus this HPLC method is only suitable for derivatised fatty acid only.

Another study that employed HPLC is performed by Zeng *et al.* (2017) on the analysis of fatty acid in *Caragana* species. Separation of fatty acid was achieved using a reversed-phase C_{18} column (250 × 4.6 mm, 5 μm) combined with a gradient elution. Elution A and B were acetonitrile, water and methanol (5:95:0.1) and 100% acetonitrile, respectively with the column temperature of 30°C and the flow rate of 1.0 ml/min. The gradient elution programme was as follows: 80%–85% B from 0 to 9.5 min; 85%–100% B from 9.5 to 18 min; and then 100% B held for 12 min. The wavelengths of fluorescence excitation and emission were set at 330 and 380 nm respectively. The identification of fatty acid derivatives was performed using an online mass spectra equipped with an atmospheric chemical ionization. Similar to the method by Tarola *et al*, (2012), this method is also suitable for derivatised fatty acid only while the method employed in our study can readily detect linoleic without having to undergo the time-consuming derivatization step. Moreover, an online mass spectra equipped with atmospheric chemical ionization were not available for the method to be adopted.

Mushrooms are rich in polyunsaturated fatty acids especially linoleic acids. A study of fatty acid analysis of nine wild edible mushrooms (*Aleurodiscus vitellinus*, *Cortinarius magellanicus*, *Hydropus dusenii*, *Cyttaria hariotii*, *Fistulina antarctica*, *F. endoxantha*, *Grifola gargal*, *Lepista nuda* and *Ramaria patagonica*) from Argentina showed that the dominant fatty acid found in the investigated species was linoleic acid, followed by oleic and palmitic acids (Toledo *et al.*, 2016). Another study on the phytochemical analysis of the *Rhizopogon luteolus* showed that eight fatty acids were detected in *R. luteolus*. Major fatty acids were linoleic (45.8%),

stearic (23.7%) and oleic acids (16.2%) (Tel-Çayan *et al.*, 2016). The fatty acid analysis of sixteen strains of *Pleurotus ostreatus* mushrooms showed that 28 fatty acids were detected in mushroom lipids. It is reported that linoleic acid is predominant in all samples (56.8–80.5%) (Koutrotsios *et al.*, 2017) indicating that it is an important constituent to be analysed in mushroom samples.

In our study, water-based extract (hot water, cold water, hexane residue and petroleum ether residue extracts) was prepared at higher concentrations (10 mg/ml) as compared to the organic solvent-based extract [hexane and petroleum ether extracts (100 µg/ml)] because during the optimisation stage, linoleic acid was undetectable in the water-based sample at concentrations lower than 10 mg/ml. One of the solvents used to extract fatty acid in this study is hexane. Hexane is widely used in extraction of natural product because of its high stability as well as low boiling point and corrosiveness (Zonouzi *et al.*, 2016). The other organic solvent used is petroleum ether which is also non-polar and can attract non-polar compounds out of the extracts.

We found that the amount of linoleic acid in water-based extracts is significantly lower than that of the organic solvent-based extracts. This is because the solvents used during the extraction process were specific for the extraction fatty acids. It is plausible that the non-polar solvents such as hexane and petroleum ether attract the long hydrocarbon alkane chain present in fatty acids. The effect was similarly shown in another study on the extraction of fatty acids from flaxseed oil where non-polar solvents such as hexane and petroleum ether fatty acids were found to be better at extracting fatty acids as compared to polar solvents (Gutte *et al.*, 2015).

Gas chromatography-mass spectrometry analysis of volatile constituents of *L. rhinocerus* by Johnathan *et al.* (2016) has shown that five major groups of constituents are present in *L. rhinocerus* including alkanes, fatty acids, benzene, phenol and dicarboxylic acid. Overall, eighteen volatile compounds were detected in five different solvents used such as hexane, petroleum ether, diethyl ether, ethyl acetate and methanol. Hexane is reported to extract the highest amount of volatile compounds (octadecane, heptadecane, 1,3-di-tert-butylbenzene, heneicosane, tricosane, 1-chlorooctadecane, tritetracontane, eicosane) while methanol extracted the lowest amount of volatile compounds (linoleic acid and ethylbenzene). Again, linoleic acid (21.35%) was found to be the major proportion of hot water extract.

4.3 Analysis of β-glucan in *L. rhinocerus* extracts

Many species of mushrooms are known for their high β-glucan contents and are well studied. *Lentinus edodes* (shiitake) and some members of the genus *Pleurotus* spp. (oyster mushrooms) are some of the most important sources of β-glucan (Rop *et al.,* 2009). β-glucan is also present in many other mushrooms. Measurement of β-glucan of three basidiomycete *Schizophyllum commune* extracts using the Mushroom and Yeast β-glucan Assay Kit (Megazyme Int.) showed that β-glucan content in the mushroom extracts ranged between 22 and 25% (Klaus *et al.*, 2011). The analysis of β-glucan in *Ganoderma applanatum*, *Ganoderma lucidum*, *Lentinus edodes* and *Trametes versicolor* using the same detection kit exhibited similar β-glucan contents at 16.0, 41.4, 41.2 and 33.4% respectively (Kozarski *et al.*, 2012).

Several reports on the analysis of β-glucan in *L. rhinocerus* have been published so far. A group of researcher from Agro-Biotechnology Institute, Malaysia

has reported that β-glucan constitute more than 50% of glucan content in *L. rhinocerus* (Mohd Jamil *et al.*, 2013) while Lau *et al.* (2013) reported that β-glucan constituted the majority [82 to 93% (w/w)] of glucan. However, another study on the β-glucans content stated that β-glucan constitute only 9% of total glucan compositions (Kong *et al.*, 2016) indicating that the concentration may vary batch to batch or based on the collection area of the mushroom. Our data indicated that β-glucans make up more than 20% of total glucan in all extracts.

Quantification of β-glucan in most studies was performed using a Megazyme Yeast and Mushroom β-glucan Assay Kit that is based on an enzymatic hydrolysis and quantification of the free sugars released. The difference in β-glucan content may be attributed to the acid used during the initial step of the procedure. In our study, sulphuric acid was used according to manufacturer's protocol but another study on the analysis of β-glucan reported the use of hydrochloric acid (Lau *et al.*, 2013; Mohd Jamil *et al.*, 2013). Another study by McCleary and Draga (2016) which compared the use of hydrochloric and sulphuric acids in acid hydrolysis step indicated that higher total and β-glucan were obtained when hydrochloric acid was used in the acid hydrolysis step. Hydrolysis of acid is very unspecific (Zhu *et al.*, 2015) and may lead to ineffective solubilization of some glucan material contributing to differences in the amount of β-glucan in our study and the other studies mentioned above. Moreover, the extraction method used in each study is different and the difference in β-glucan content is probably attributed to the different extraction method employed.

4.4 Cell studies

4.4.1 BEAS-2B

Bronchial epithelial cells play an important role in conserving a normal airway function. They act as a defensive barrier by forming an interface between the external and the internal environment of human lungs, making it a prominent target of airways insults (Schleimer and Berdnikovs, 2017). They also act as effectors in inflammatory responses by secreting chemokines and cytokines that may eventually activate inflammatory cells (Gao *et al.*, 2015). Hence, BEAS-2B, an *in vitro* pulmonary model was chosen for the *in vitro* part of this study.

BEAS-2B is a human lung epithelial cell line immortalized by the SV40 large T-antigen and was isolated from the normal human bronchial epithelium of a cancer-free individual. BEAS-2B is widely used as a pulmonary epithelium model in many types of *in vitro* studies including toxicology testing, respiratory injury and remodelling (Anthérieu *et al.*, 2017). To date, there is no uniform culture conditions specific for BEAS-2B reported. Both serum-free supplemented media are used while the culture media reported for BEAS-2B include serum free bronchial epithelial cell growth medium (Kale *et al.*, 2017) and Dulbecco's modified Eagle's medium with foetal bovine serum supplementation (Lv *et al.*, 2017).

4.4.2 Cell proliferation assay

The proliferative activity of BEAS-2B treated with various concentrations of *L. rhinocerus* extracts was measured using an MTS assay. MTS assay is a colourimetric method used to assess the metabolic activity of cells. The assay is measured by the rate of reduction of tetrazolium salt into coloured formazan in the presence of nicotinamide adenine dinucleotide phosphate produced by

dehydrogenase enzymes in the living cells (Berridge and Tan, 1993). Since dead cells lose the ability to reduce tetrazolium salts, the formation of formazan is directly proportional to the living cells. However, unlike MTT, the formazan products from the reduction of MTS are soluble in the culture medium. Hence, this assay is more convenient than MTT because a second addition of reagent to solubilize formazan precipitates is not required.

From the data obtained, all extracts especially at 125 and 250 μg/ml showed reduction in the proliferative activity as compared to the control at 72 hours although the majority of the extracts is not cytotoxic against BEAS-2B cell line. The data is consistent with a study on the anti-proliferative effect of the cold water extract from sclerotia of *L. rhinocerus* against MCF-7 and A549 cells (Lee *et al.*, 2012), suggesting that the majority of the extract is not toxic to BEAS-2B cell line. According to the International Organization for Standardization Test for *in vitro* cytotoxicity, cytotoxity can be quantitatively evaluated by measuring cell death, inhibition of cell growth and proliferation. A substance is considered as cytotoxic when the reduction of cell viability is more than 30% (ISO, 2009). In this study, cold water and petroleum ether extracts show cytotoxic effect only in high concentration (62.5 – 250.0 μg/ml) since the reduction of cell viability at 72 hours is more than 30%.

Lau *et al.* (2013) hypothesized that the cytotoxicity effect of cold water extract is due to thermolabile compounds that present in cold water extract but absent in hot water extract. Protein profiling coupled with the MTT assay was performed to prove the hypothesis. The study reported that cytotoxic cold water extracts contained more proteins than non-cytotoxic hot water extract, suggesting cytotoxic activity may be attributed to the additional proteins in the cold water extract. In addition, the loss

of cytotoxicity from heat-treated cold aqueous extracts was accompanied by changes in protein profiles corresponding to the denaturation of proteins at temperatures higher than 60^{o}C.

In addition, the content of each extract varies due to the type of solvent and extraction methods used. Based on the review by Tiwari *et al.* (2011) on phytochemical screening and extraction, water has been used to extract anthocyanins, starches, tannins, saponins, terpenoids, polypeptides and lectins. Although water is used in both hot and cold water extracts, it is possible for the two extracts to have disparity in the constituents yielded because hot water may be able to pull out higher number of substance. Ether, on the other hand has the ability to extract alkaloids, terpenoids, coumarins and fatty acids. Many plants that are reported to have toxic properties usually contain certain terpenoid and alkaloids (Wink, 2015). Wink (2015) suggested that both cold water and petroleum ether extracts pull terpenoids out of the natural product. In addition, petroleum ether extract may also attract alkaloids. The toxic properties of the extracts have been attributed to the presence of bioactive compounds such as terpenoids and alkaloids.

4.4.3 Apoptosis assay

Apoptosis is characterized by several morphological changes including translocation of membrane phospholipid phosphatidylserine, condensation of nucleus and cytoplasm as well as DNA cleavage (Berghe *et al.*, 2013). At the early stage of apoptosis, the cells lose their membrane integrity due to the translocation of phospholipid phosphatidylserine from the inner to the outer side of plasma membrane resulting in the exposure of phospholipid phosphatidylserine to external environment (Orrenius *et al.*, 2010). A phospholipid-binding protein, Annexin V

(conjugated with fluorochromes) can serve as a marker for the detection of apoptotic cells because it to the phospholipid phosphatidylserine on the outer leaflet of apoptotic cell (Wlodkowic *et al.*, 2009). The translocation of phospholipid phosphatidylserine will eventually lead to the loss of membrane integrity which is also the result of necrosis. Thus, to identify the cells, Annexin V is combined with propidium iodide to distinguish the different stages of apoptotic cells (Rieger *et al.*, 2011).

Viable cells do not stain with Annexin V and propidium iodide because their membranes are still intact and is nonpermeable to propidium iodide. However, in the early and late apoptotic cells, the membranes are damaged and permeable to propidium iodide. Therefore, early apoptotic cells are Annexin V positive and propidium iodide negative, while cells in the later stage are both Annexin V and propidium iodide positive. The necrotic cells will also be stained with both Annexin V and propidium iodide (Pietkiewicz *et al.*, 2015). Hence, it is difficult to differenciate between late apoptotic cells and necrotic cells. However, when apoptosis is measured over time, cells can often be tracked from viable to early apoptosis and end stage apoptosis (Tominami *et al.*, 2015).

In a previous study Fauzi *et al.* (2015), *L. rhinocerus* is reported to induce apoptosis in HCT 116 colon cancer cells. *L. rhinocerus* was also found to induce apoptosis that lead to cell death in the human breast carcinoma cell line, MCF-7 and the human lung carcinoma cell line, A549 (Lee *et al.*, 2012). In our study, however, statistical analysis indicated that treatment with *L. rhinocerus* extracts do not have any effect on apoptotic activity of BEAS-2B. It is plausible that the type of cell used in this study, which is the normal cell may cause the variation in the findings. It is important to know that treatment with *L. rhinocerus* extracts does not induce

apoptosis in normal cell disregulation of apoptosis in normal cells can result in a number of diseases such as cancers, autoimmune diseases, inflammatory diseases, and viral infections. Our findings indicate the safety of *L. rhinocerus* and its potential use of this extracts for therapeutic purposes.

4.4.4 Cytokine profile analysis

Cytokines are small proteins that regulate the immune response. Cytokines involved in cell communication, growth and survival. They also induce gene expression. Many types of cell involved in cytokine production. For instance, CD4+ helper T-cells generate large amounts of cytokines with distinctive function in the course of the adaptive immune response. These helper cells can become T-helper 1 cells which generate large amounts of IFN-γ or they may also turn into T-helper 2 cells making several types of IL such as IL-4, IL-5, and IL-13. In addition, these helper cells can also become T-helper 17 cells producing high levels of IL-17. These cytokines act differently in lung diseases including asthma, chronic obstructive pulmonary disease, and pulmonary fibrosis. While each disease has distinguished characteristics, only several cytokines (TGF-β, IL-17, IL-4, IL-13) play major roles in all three diseases.

Hu *et al.* (2017) performed a study on the effect of polysaccharides fractions of *L. rhinocerus* on T-helper 1 cytokines of immunosuppressive mice model indicated that the four *L. rhinocerus* fractions at 20 mg/kg/d significantly improved serum TNF-α and INF-γ levels, while no noticeable change was detected on serum IL-10 production. In a separate study on the effect of *L. rhinocerus* on T-helper 2 cytokines, hot water extract of *L. rhinocerus* significantly reduced IL-4, IL-5 and IL-13 levels in bronchoalveolar lavage fluid of ovalbumine-induced asthma model

indicating its efficiency in reducing inflammation (Johnathan *et al.*, 2016). However, in contrast to significant effect shown in the previous studies, the data obtained from this study showed that *L. rhinocerus* did not give any significant effect to the cytokine production in normal BEAS-2B perhaps due to the different model used in the study. While the previous studies were using induced *in vivo* models, our study was performed *in vitro* using non-targeted cells.

4.5 Limitations of the study

This thesis describes *in vitro* studies of the potential role of *L. rhinocerus* on BEAS-2B. However, there are some limitations to the extraction method used in this study. The extraction method utilised only on linoleic acid extraction and water-based extracts. Secondly, although detection of linoleic as well as quantification of β-glucan was conducted, chemical profile of the extract is lacking and therefore the presence of other bioactive compound in each extract is unknown.

4.6 Future study

Research on the therapeutic properties of *L. rhinocerus* is still progressing with many areas remained unexplored. Identification and isolation of bioactive components that are believed to play roles in certain diseases are needed to validate the therapeutic properties of *L. rhinocerus*. Since *L. rhinocerus* is still widely used traditionally, a thorough investigation on the chemical profiles of crude extracts and their therapeutic effects should be reported to ensure the safety and efficacy of the extracts for traditional use. The investigation can also validate the traditional preparation for quality control of large-scale preparations. Since previous studies are mostly focused on the beneficial effects of the sclerotium, investigation on other parts of *L. rhinocerus* which may have some therapeutic properties is still lacking. In

addition, mushrooms might produce either different active ingredients or similar active ingredients in different amounts at different developmental stages (Lau *et al.*, 2015) and therefore constant research is needed.

Previously, the extraction method employed in this study only focused on the production of extracts rich in linoleic acid composition (hexane and petroleum ether extracts) because investigation on the potential effect of linoleic on respiratory diseases was warranted. Future study should be adapted *in vivo* models such as asthma and chronic obstructive pulmonary disease. In addition, future study should also focus on another type of fatty acid which is omega-3 fatty acid that also has many therapeutic effects on respiratory diseases. In addition, a specific method to extract β-glucan should be employed because β-glucan also has many potential on respiratory diseases based on previous literatures. Since some of the extract shows antiproliferative effect on BEAS-2B, chemical profiling analysis of the extract is required while investigation on cancerous lung cell line should be done as well to determine the potential of *L. rhinocerus* on lung cancer.

CHAPTER 5

CONCLUSION

Quantification of linoleic acid in hot water, cold water, hexane, petroleum ether, hexane residue and petroleum ether residue extracts of *L. rhinocerus* has been successfully determined using HPLC. Petroleum ether was found to be the best solvent to extract linoleic acid. In addition, the amount of β-glucan in *L. rhinocerus* extract has been successfully quantified using the optimised method. Hexane residue extracted the highest concentration of β-glucan (39.8 g) while petroleum ether yielded the least (24.1 g). The effects of these extracts on BEAS-2B bronchial epithelial cell line were investigated. All extracts (especially at high concentration, 125 and 250 μg/ml) showed reduced proliferative activities as compared to control at 72 hours; although the majority of the extracts were not cytotoxic to BEAS-2B cell line. Moreover, *L. rhinocerus* has no effect on the apoptotic activity as well as on the T-helper 1/T-helper 2/T-helper 17 cytokines release indicating the potential use of the extracts for future study.

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APPENDICES

Appendix A: List of publications

Publications under review

1. **Siti Nurshazwani Muhamad Sayuti,** Gan Siew Hua and Nurul Asma Abdullah. Fatty acids, their eicosanoids and asthma. *Food Bioscience*.

2. **Siti Nurshazwani Muhamad Sayuti,** Gan Siew Hua and Nurul Asma Abdullah. Detection of linoleic acid and β-glucan in *L. rhinocerus* extracts and the biological properties of the extracts on BEAS-2B cell line. *Chromatographia*.

Other publication

1. Tan, TK, Johnathan, M, **Muhamad Sayuti**, **SN**, and Nurul AA. Immunomodulatory properties of tualang honey in BALB/c mice. Publication in Research Journal of Pharmaceutical, Biological and Chemical Sciences 2015.

Abstract of publications under review

Fatty acids, their eicosanoids and asthma

Authors: Siti Nurshazwani Muhamad Sayuti[1], Siew Hua Gan [2] & Asma Abdullah Nurul[3]

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Abstract

Fatty acids with the help of rate limiting enzymes such as cyclooxygenase (COX), lypoxygenase (LOX) and cytochrome P450 monooxygenase (CYP) are involved in the generation of lipid mediators which responsible in the pathogenesis of asthma. Lipid mediators produced from arachidonic acid such as Prostaglandin D_2 (PGD_2), Cysteinyl leukotrienes (Cys-LTs) and Leukotriene B_4 (LTB_4) promote inflammation whereas omega-3 derived lipid mediators such as protectins, resolvins and maresins are said to have protective effects against inflammatory diseases including asthma and allergy. These lipid mediators which are better known as special preresolving lipid mediators (SPM) are vital components in the resolution of inflammation. There are reports on the impairment of SPM biosynthesis especially in severe asthma suggesting that chronic inflammation in the lung is probably due to a resolution defect. In this review, the roles of fatty acids in asthma, particularly omega-3 and omega-6 are discussed.

Keywords: asthma; eicosanoids; fatty acid; lipid mediators; omega-3; omega-6

Detection of linoleic acid and β-glucan in *L. rhinocerus* extracts and the biological properties of the extracts on BEAS-2B cell line.

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Abstract

A simple high performance liquid chromatographic (HPLC) method was optimised for detection of linoleic acid in *L. rhinocerus*. Separation was achieved on reverse-phase C18 column (250 × 4.6 mm, 5μm) and the detection was monitored by UV detector at 208 nm. The optimized mobile phase used was the combination of acetonitrile, methanol and hexane in the ratio of 90:8:2 with the addition of 0.2% acetic acid at a flow rate of 1.0 ml/min. Analysis of β-glucan composition in water-based *L. rhinocerus* extracts indicated that hexane residue is the best extract for β-glucan. In this study, cold water and petroleum ether extract at the concentration of 62.5-250 μg/ml show cytotoxic effect as the reduction of cell viability at 72 hours is more than 30% while the other extracts are not cytotoxic. Statistical analysis shows that the treatment with *L. rhinocerus* extracts does not have any effect on the apoptosis rate of BEAS-2B.

Keywords: Tiger milk mushroom, *lignosus rhinocerus*, linoleic acid, HPLC

Other publication

ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Immunomodulatory Properties of Tualang Honey in BALB/c Mice.

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ABSTRACT

Local natural products have been gaining great focus from research and therapeutic aspects. The outcome of studies on one of nation's renowned Tualang honey (TH) (honey bee collected from *Koompassia excels* tree) has profoundly anchored its broad aptitude in anti-inflammatory, anti-oxidant and anti-microbial properties. This study was aimed to investigate the immuromodulatory properties of TH in BALB/c mice. TH was orally administered daily for 14 days to male BALB/c mice (5/group) in dose ranging from 0.5 g/kg, 1.5 g/kg and 3.0 g/kg per group. Pre-treatment and post-treatment body weights were measured. Upon sacrificing, the spleen was weighed and then homogenized. The splenocytes were stained with various surface markers antibodies namely $CD3^+/CD4^+$ (T helper), $CD3^+/CD8^+$ (T cytotoxic), $CD14^+$ (macrophage) and CD19+ (B lymphocyte) and immune cell populations were obtained by using flow cytometer. On the other hand, proliferation assay of splenocytes were done by using CelTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Body weight of mice showed increment after the treatment but body weight of mice treated with 0.5 g/kg decreased upon completion of TH treatment. BALB/c mice treated with TH showed increased populations of $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD14^+$ and $CD19^+$ compared to the control group. Proliferation analysis of splenocytes and spleen weight obtained from TH-treated mice also showed increment. The results revealed the immunostimulant potential of TH in mice by enhancing lymphocyte populations especially T helper ($CD3^+/CD4^+$), cytotoxic ($CD3^+/CD8^+$) and B ($CD19^+$) cells.

Keywords: immunomodulation, natural product, food, Tualang honey

Appendix B: List of presentations

Poster presentation:

1. **Siti Nurshazwani Muhamad Sayuti,** Gan Siew Hua and Nurul Asma Abdullah. Cytotoxicity activity of *Lignosus rhinocerus* on human bronchial epithelial lung cell line (BEAS-2B). 3rd Pan-Asian Biomedical Science Conference 2016, Kuala Lumpur, Malaysia.

Oral presentation:

1. **Siti Nurshazwani Muhamad Sayuti,** Gan Siew Hua and Nurul Asma Abdullah. Determination of linoleic acid and β-glucan compositions in Tiger Milk Mushroom (*Lignosus rhinocerus*). Health Sciences Symposium 2017, Kota Bharu, Kelantan Malaysia.

Other presentations

Poster presentations:

1. **Siti Nurshazwani Muhamad Sayuti**, Johnathan Malagobadan, Jamaruddin Mat Asan and Nurul Asma Abdullah (2013). Immunomodulatory properties of virgin coconut oil in BALB/c mice. International Conference of Medical and Health Science 2013, Kota Bharu, Kelantan, Malaysia.

2. Tan Tiong Kit, Johnathan Malagobadan, **Siti Nurshazwani Muhamad Sayuti**, Jamaruddin Mat Asan and Nurul Asma Abdullah. Immunomodulatory properties of Tualang honey (Koompassia excels) in BALB/c mice. International Conference of Medical and Health Science 2013, Kota Bharu, Kelantan, Malaysia.

Abstract in conferences

Poster presentation at the 3rd Pan-Asian Biomedical Science Conference 2016
Kuala Lumpur, Malaysia
7-8 December 2016

Cytotoxicity activity of *Lignosus rhinocerus* on human bronchial epithelial lung cell line (BEAS-2B)

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Abstract

Lignosus rhinocerus or better known as Tiger Milk Mushroom (TMM) is one of the medicinal mushrooms used by indigenous people of Southeast Asia and China to treat various diseases. In Malaysia, it is the most popular medicinal mushroom used by the indigenous communities to relieve fever, cough, asthma, cancer, food poisoning and as a general tonic. BEAS-2B cells (2×10^3 cells per well) were treated with four different extracts of TMM namely hot water, cold water, hexane and petroleum ether extracts with the concentrations of 250, 125, 62.5, 31.25 and 15.625 µg/ml. The cells were treated with the extracts for 3 days respectively. Analysis of cytotoxicity was carried out by MTS assay. The extracts of TMM were evaluated for their cytotoxicity against the BEAS-2B cell line at five different concentrations. The results of the present study indicated that the cell viability decreased with increase in the concentration of each TMM extracts. The highest inhibition was recorded at the concentration of 250 µg/ml. Among the extracts, TMM extracted by hot water was recorded the highest viability activity. In contrast, cells treated with 250 µg/ml petroleum ether extract recorded the highest inhibition effect where the viability percentage was <20%. Lack of cytotoxicity activity represents by hot water, cold water and hexane methods indicated that these methods are suitable for downstream studies.

Keywords: *Lignosus rhinocerus*; Tiger Milk Mushroom; cytotoxicity; mushroom

Oral presentation at the Health Sciences Symposium 2017
Kota Bharu, Kelantan, Malaysia
25 May 2017

Determination of linoleic acid and β-glucan compositions in Tiger Milk Mushroom (*Lignosus rhinocerus*)

Siti Nurshazwani Muhamad Sayuti[1], Gan Siew Hua[2] and Nurul Asma Abdullah[3]

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Abstract

Introduction: *Lignosus rhinocerus* or Tiger Milk Mushroom (TMM) is one of the medicinal mushrooms used by indigenous people of Southeast Asia and China to treat various diseases including fever, cough, asthma, cancer and food poisoning. Mushrooms are considered healthy because they contain various nutraceutical compounds including polysaccharides and unsaturated fatty acids. This study is aimed to quantify linoleic acid and beta-glucan (β-glucan) from TMM which extracted using various methods.
Methods: TMM was extracted using four methods i.e. hot water, cold water, hexane and petroleum ether. Measurement of linoleic acid in these extracts was performed using high performance liquid chromatography (HPLC) while quantification of β-glucan was done using Mushroom and Yeast β-glucan Assay Kit.
Results: The amount of linoleic acid in water-based extracts such as hot water, cold water, hexane residue and petroleum ether residue extracts were 0.05 g in 100 g of extracts respectively. While the amount of linoleic acid in extracts that were extracted using non-polar solvents such as hexane and petroleum ether were 16.79 g and 17.55 g in 100 g of extract respectively. Analysis of β-glucan composition in water-based TMM extracts indicated 29.0 g, 33.6 g, 39.8 g and 32.1 g in 100 g extracts of hot water, cold water, hexane residue and petroleum ether residue extracts respectively, while the amount of β-glucan in non-polar extracts such as hexane and petroleum ether extracts were 31.5 g and 24.1 g respectively.
Conclusion: In conclusion, non-polar solvent extracts yield more linoleic acid compare to water-based extracts while the yield of β-glucan composition is not affected by the extraction methods used in the study.

Keywords: Beta-glucan, HPLC, *Lignosus rhinocerus*, linoleic acid, tiger milk mushroom

Appendix C: Certificate of analysis (*L. rhinocerus*)

LiGNO™ Biotech Sdn Bhd (839988-X)
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Website:ww.ligno.com.my

PRODUCT SPECIFICATION

LiGNO™ TM02 Freeze Dried Powder

Scientific Name	:	*Lignosus rhinocerus* cultivar TM02
Common Name	:	Tiger Milk Mushroom; Cendawan Susu Harimau; 虎乳芝
Part	:	Sclerotia
Shelf Life	:	3 years

Test Parameter	Method/Reference	Specification
Physical Properties		
Color	In-house	Light brown
Odor		Characteristic
Flavor		Characteristic
Form/Texture		Fine Powder
pH		5.5 -6.5
Moisture Content		< 10%
Heavy Metal		
Lead (Pb)	AOAC	≤ 10 ppm
Mercury (Hg)		≤ 0.5 ppm
Arsenic (As)		≤ 5 ppm
Cadmium (Cd)		≤ 0.3 ppm
Microbial Limit Tests		
Total Aerobic Plate Count	BP2002	≤ 2 x 10^4 cfu/g
Yeast & Mold		≤ 2 x 10^2 cfu/g
Enterobacteria and certain other Gram –ve bacterial		≤ 2 x 10^2 cfu/g
Salmonella		Absent in 10 g
Escherichia coli		Absent in 1 g
Staphylococcus aureus		Absent in 1 g

LiGNO™ Biotech Sdn Bhd (839988-X)
Fax / Tel: 603 8945 5 242
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Website:ww.ligno.com.my

HPLC Profiling Test

Test Parameter : Peak 1 (P1) and Peak 2 (P2)

Method/Reference : In-house

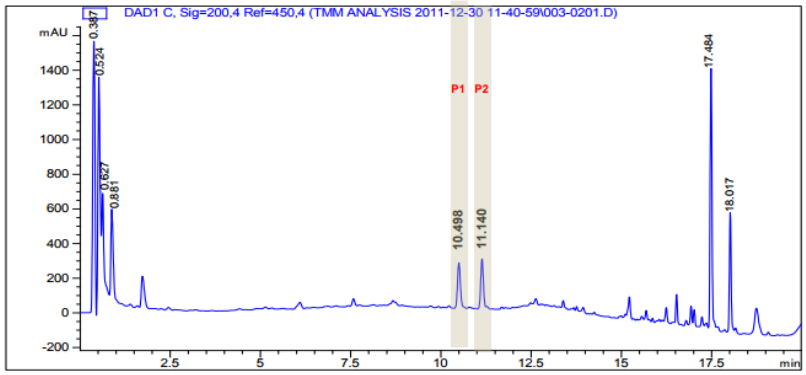
Specification :

i. P1 & P2 must be detected simultaneously.

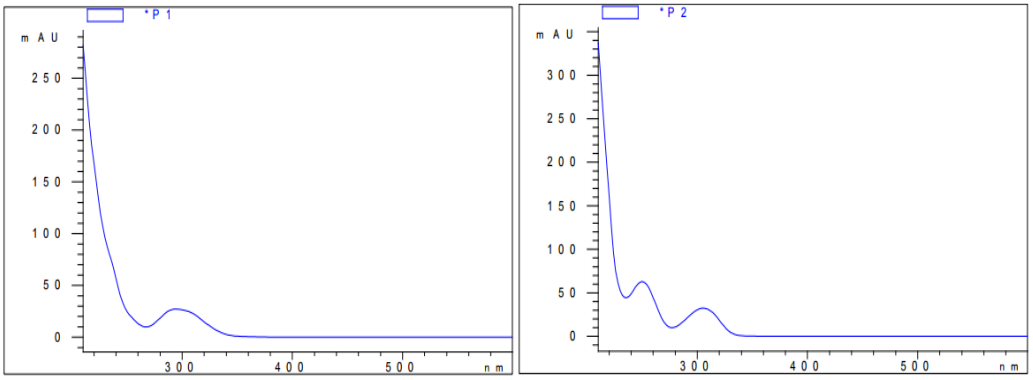
ii. Retention time of P1 & P2 must not deviate more than ±5% from the reference standard.

iii. UV spectra of P1 & P2 must match with the UV spectra of the reference standard.

1. HPLC chromatograms of LiGNO™ TM02 Freeze-dried powder



2. UV Spectra of P1 and P2



Appendix D: Certificate of analysis (linoleic acid)

Version	0
Molecular weight	280.45
Molecular formula	C18 H32 O2
CAS No	60-33-3
Linear formula	CH3(CH2)4CH=CHCH2CH=CH(CH2)7CO2H

Certificate of Analysis

This is to certify that units of the below mentioned lot number were tested and found to comply with the specifications of the grade listed. Certain data have been supplied by third parties. Acros Organics expressly disclaims all warranties, expressed or implied, including the implied warranties of merchantability and fitness for a particular purpose. Unless otherwise stated, these products are not intended for dialysis, parenteral or injectable use without further processing. The following are the actual analytical results obtained:

Catalog Number	21504	**Quality Test / Release Date**	14 February 2014
Lot Number	A0344296	**Suggested Retest Date**	February 2017
Description	Linoleic acid,99%		
Country of Origin	SWEDEN		
Declaration of Origin	plant		

Origin Comment	extracted from sunflower and safflower oil

Result Name	Specifications	Test Value
Appearance	clear colorless to light yellow liquid	clear colorless liquid
Infrared spectrum	authentic	authentic
GC	>=98.5 %	99.1 %
Refractive index	1.4687 to 1.4707 (20°C, 589 nm)	1.4702 (20°C, 589 nm)

L. Van den Broek, QA Manager

Issued: 23 August 2017

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